Synthesis of Modified Oligonucleotides

by

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To

My Parents

and

Susanne

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Abbreviations.

A	Adenosine
AAAF	N-acetoxy-N^2-acetylamino fluorene
1,5-AEDANS	5-(2-(Iodoacetyl)aminoethyl)-aminonaphthalene-1-sulphonic acid
AMPPD	Adamantyl monopyridinium phosphate dioxetane
AP	Alkaline Phosphatase
ATP	Adenosine triphosphate
AZT	3'-Azido-3'-deoxythymidine
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
Bio	Biotin
C	Cytidine
CD4	Cell surface receptor recognised by HIV gp120 glycoprotein
Chol	Cholesterol
CPG	Controlled-pore glass
dA	2'-Deoxyadenosine
DAB	3, 3'-Diaminobenzidine
dC	2'-Deoxycytidine
DCC	N,N'-Dicyclohexyl carbodiimide
dG	2'-Deoxyguanosine
Dig	Digoxigenin
DMSO	Dimethyl sulphoxide
DMTr	4, 4'-Dimethoxytrityl
DNFB	2, 4-Dinitrofluorobenzene
DNP	2, 4-Dinitrophenyl
dNTP	2'-Deoxynucleoside triphosphate
D-ODN	Normal phosphodiester-linked oligodeoxynucleotide
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
dU	2'-Deoxyuridine
dUTP	2'-Deoxyuridine triphosphate
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid

FAM	Applied Biosystems trade name for fluorescent dye shown in *Figure 3* of Chapter 1.
FITC	Fluorescein isothiocyanate
Fmoc	9-Fluorenylmethyloxycarbonyl
G	Guanosine
gp120	HIV glycoprotein involved in viral recognition of cells
heg	hexaethyleneglycol
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
JOE	Applied Biosystems trade name for fluorescent dye shown in *Figure 3* of Chapter 1.
K3	Code name for *anti*-DNP antibody
kDa	kiloDalton
LDL	Low-density lipoprotein
Lys-ODN	poly-(L-Lysine)-conjugated oligodeoxynucleotide
MP-ODN	Methylphosphonate oligodeoxynucleotide
mRNA	Messenger ribonucleic acid
NAP	Nucleic Acid Purification (Sephadex G-25)
NBT	Nitro blue tetrazolium
NHS	N-Hydroxysuccinimide
ODN	Oligodeoxynucleotide
p	Phosphate
p24	HIV glycoprotein
PAC	Phenoxyacetyl
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
Phth	Phthaloyl
PL-ODN	Phospholipid oligodeoxynucleotide
RNase H	Ribonuclease H
ROX	Applied Biosystems trade name for fluorescent dye shown in *Figure 3* of Chapter 1.
S-ODN	Phosphorothioate oligodeoxynucleotide
T	Thymidine
TAMRA	Applied Biosystems trade name for fluorescent dye shown in *Figure 3* of Chapter 1.

TETD	Tetraethylthiuram disulphide
THF	Tetrahydrofuran
THP	Tetrahydropyranyl
Ts or Tosyl	*para*-Toluene sulphonyl
Tween-20	Polyoxyethylenesorbitan monolaurate
UTP	Uridine triphosphate
VitE	Vitamin E

5'----------3'

or A single-stranded oligonucleotide

3'----------5'

Abstract.

The 2,4-Dinitrophenyl (DNP) group is a hapten with potential use as a simple, inexpensive non-radioactive labelling group for oligonucleotides. DNP phosphoramidites have been synthesized which allow the attachment of multiple spaced and unspaced DNP groups to oligonucleotides during solid-phase DNA synthesis. Results of Polymerase Chain Reaction and antibody binding experiments are reported.

A phosphoramidite to introduce multiple hydroxyl functions into oligonucleotides has been synthesized. Reaction of the multiple hydroxyl functions with single-addition biotin and DNP phosphoramidites during solid-phase synthesis allows the introduction of multiple biotin or DNP labels onto the oligonucleotides in high yield.

Two single-addition biotin phosphoramidites have been synthesized incorporating benzoyl protection of the biotin moiety, and different spacer arm lengths. These were used for the synthesis of biotinylated oligonucleotides during solid-phase synthesis.

In an attempt to increase cellular uptake of antisense oligonucleotides Vitamin E, cholesterol and adamantane have been attached to oligonucleotides during solid-phase synthesis. Results of thermal denaturation studies on lipophilic oligonucleotides are reported.

Contents.

General Introduction

The automated synthesis of oligonucleotides using solid-phase methodology has revolutionized many areas of medicine, molecular biology and biotechnology. The potential use of modified oligonucleotides as highly sensitive tools for the diagnosis of inherited and other diseases, and as 'antisense' pharmaceuticals[1] for the treatment of viral diseases and cancer, continues to fuel interest in this field.

Unlike solution-phase synthesis, the solid-phase approach precludes the need to purify intermediates during oligonucleotide assembly. This allows the rapid and inexpensive automated synthesis of oligonucleotides up to *ca.* 150 nucleotides long. Oligonucleotides which would have taken several months to synthesize by solution-phase methods can be synthesized in 24 hours by solid-phase methodology. Unlike enzymatic synthesis, chemical synthesis allows specific modifications to be made to the oligonucleotide at specific sites in a very controlled manner. Such modifications include: the insertion of unnatural and rare bases at specific sites; modification of the phosphodiester backbone; and the attachment of non-nucleosidic residues, such as non-radioactive labels, to the oligonucleotide.

A full review of the development of oligonucleotide synthesis is given in a recent review by Beaucage and Iyer[2]. The organic chemistry underlying oligonucleotide synthesis is described in an excellent review by Sonveaux[3].

By far the most widely used method of solid-phase oligonucleotide synthesis at present is the phosphoramidite method. This allows the very rapid synthesis of oligonucleotides (*ca.* 7 minutes per synthesis cycle) in exceptionally high yields (>98.5% per synthesis cycle) on a controlled-pore glass or polystyrene solid phase. Standard oligonucleotide synthesis

and purification procedures using the phosphoramidite method are described in detail by Brown and Brown[4].

The work described in this thesis had the following general aims:
(1) To introduce modifications to oligonucleotides using solid-phase phophoramidite methodology.
(2) To design and synthesize compounds of practical and potential commercial use to introduce these modifications. This entails the use of inexpensive, commercially available starting materials and as few synthetic steps as possible to reach the target compounds.
(3) The introduction of the modifications should require little or no change to standard oligonucleotide synthesis and purification conditions. The practicality and commercial viability of any potentially useful compound is severely impaired if the end-user must make major alterations to standard DNA synthesis and purification procedures.

This thesis is divided into two chapters:
-Chapter One describes work carried out on the non-radioactive labelling of oligonucleotides during solid-phase synthesis.
-Chapter Two describes the modification of oligonucleotides with lipophilic groups in an attempt to enhance cellular uptake of 'antisense' oligonucleotides.

The general approach - the attachment of hydrophobic, non-nucleosidic groups to the ends of oligonucleotides during solid-phase synthesis - is essentially the same in both areas. To enhance readability each chapter has its own introduction, results and discussion, experimental section and references.

References.

1. Uhlmann, E. and Peyman, A. (1990) *Chem.Rev.* **90** , 543-584.

2. Beaucage, S.L. and Iyer, R. (1992) *Tetrahedron* , **48**, 2223-2311.

3. Sonveaux, E. (1986)*Bioorganic Chem.* **14**, 274-325.

4. Brown, T. and Brown, D.J. in *Oligonucleotides and Analogues-a Practical Approach.* (1991) Eckstein, F. (Ed.); IRL Press; Oxford, 1-24.

Chapter One

The Non-radioactive Labelling of Oligonucleotides during Solid-Phase Synthesis.

A. INTRODUCTION

Non-radioactive labelling of oligonucleotides has attracted much interest in recent years as the number of biological and biomedical techniques requiring labelled oligonucleotides has increased. The use of non-isotopic labels is especially desirable in applications where a large number of similar experiments are carried out on a routine basis. Such applications include; screening of clinical samples for viral infections or genetic diseases, and large-scale automated DNA sequencing. The latter application has increased in importance recently with the advent of the 'Human Genome Project'.[1]

The most common technique used for the labelling of oligonucleotides is the introduction of a ^{32}P radiolabel by the action of polynucleotide kinase and γ-^{32}P-dATP. Other radioisotopes such as ^{35}S, ^{14}C, ^{3}H and ^{125}I can also be used.[2] The radiolabelled DNA is detected using autoradiography. This technique is extremely sensitive, but has several major disadvantages.

(i) The ^{32}P radioisotope has a half-life of 14.2 days. This requires experiments to be carried out very soon after the DNA is labelled. Thus repeated labelling of DNA is necessary for long-term experiments. This makes this type of labelling time consuming, expensive and hazardous due to repeated exposure of the experimentalist to ionising radiation. It

also introduces an element of variability into experiments, as the specific activity of the radiolabelled DNA varies with time and batch.
(ii) The cost and time required to ensure statutory shielding, monitoring, containment, storage, waste disposal and training is often high.
For these reasons several methods for the non-radioactive labelling of oligonucleotides have been developed.

1.0. General Principles of Non-Radioactive Labelling.

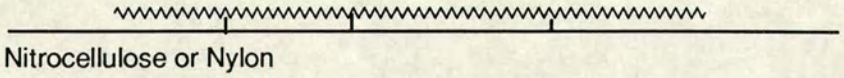
There are two basic strategies for the non-radioactive labelling of oligonucleotides:

1.1. *Directly detectable labelling.*

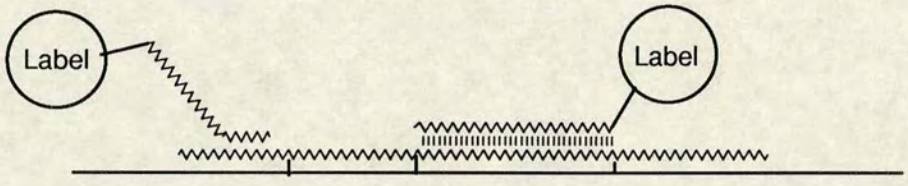
This involves the covalent attachment of a detectable molecule (such as a fluorescent dye), or an enzyme (such as alkaline phosphatase or horseradish peroxidase), to the oligonucleotide (*Figure 1*). Fluorescent groups can be detected visually by means of a fluorescence microscopy or fluorimetry. Enzymes can be detected by addition of an enzyme substrate which produces either a coloured product (which can be detected visually or spectrophotometrically), or a light signal *via* a chemiluminescent reaction (which is detected photographically or by a charge-coupled device).

The enzymes which are most commonly used at present are alkaline phosphatase (AP) and horseradish peroxidase (HRP). There are a variety of substrates commercially available for both enzymes.

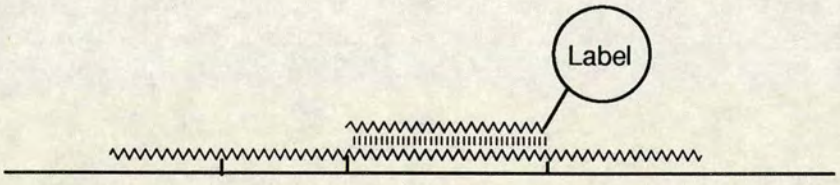
(1) Immobilise Target DNA or RNA on Membrane



(2) Hybridise with Directly Labelled Oligonucleotide Probe



(3) Wash away Non-specifically Bound Oligonucleotide Probe



(4) Detect
If Label is a Fluorophore
-detect by irradiation with light of the appropriate frequency

If Label is an Enzyme
-add enzyme substrate to the membrane to produce a coloured precipitate or chemiluminescence

Figure 1: Schematic diagram of a typical hybridisation experiment using a directly detectable labelled oligonucleotide probe. (not to scale)

1.2. *Indirectly detectable labelling.*

This involves the attachment of a molecule (such as a hapten), which is not directly detectable, to the oligonucleotide. Incubation of the hapten-labelled oligonucleotide with an enzyme-linked antibody to the hapten results in the attachment of the enzyme to the oligonucleotide (*Figure 2a*). Subsequent addition of an enzyme substrate gives rise to the signal, as described in section 1.1. above. It is desirable to attach the labelling molecule to the oligonucleotide by means of a spacer arm to minimise steric hindrance to antibody-hapten binding.[3,4] *Figure 2b* shows the relative sizes (approximately to scale) of an IgG antibody and a 20mer oligonucleotide. A variety of methods for increasing the number of enzymes bound to the oligonucleotide, and thus the signal intensity and sensitivity of detection, are possible using indirect labelling. One such method is the use of a primary antibody which binds to the labelling molecule, followed by incubation with a secondary antibody-enzyme conjugate which binds to the primary antibody. The secondary antibody can bind to several sites on the primary antibody, resulting in the indirect attachment of several enzyme molecules to the oligonucleotide.(*Figure 2*)

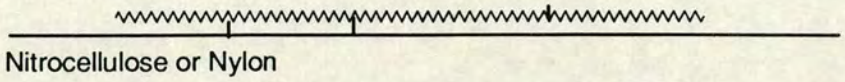
2.0. Types of labelling groups and methods of detection

2.1. *Directly Detectable Labelling Groups.*

2.1.1. *Fluorescent dyes.*

Oligonucleotides can be covalently labelled with a range of fluorescent dyes such as fluorescein, tetramethylrhodamine, Texas Red, FAM, ROX, TAMRA, JOE, dansyl and acridine derivatives(*Figure* 3). These dyes are usually attached by reaction of the dye active ester or isothiocyanate with an amino-modified oligonucleotide, or by reaction of

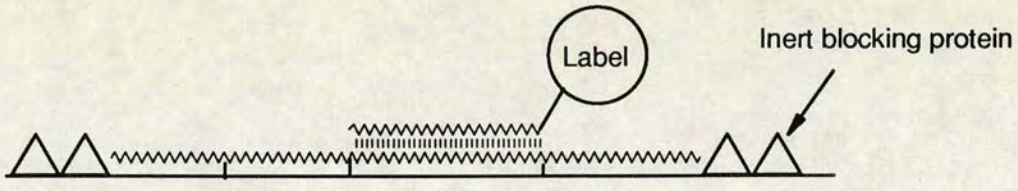
(1) Immobilise Target DNA or RNA on Membrane



(2) Hybridise with Hapten Labelled Oligonucleotide Probe

(3) Wash away Non-specifically Bound Oligonucleotide Probe

(4) Block Protein Binding Sites on Membrane



(5) Incubate with anti-hapten antibody,
then with secondary antibody-enzyme conjugate

(6) Add enzyme substrate-coloured precipitate or chemiluminescence

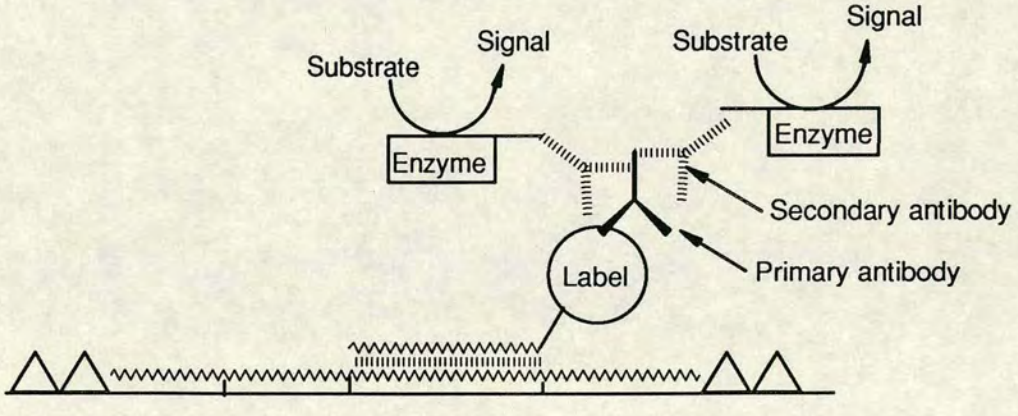


Figure 2a: Schematic diagram of a typical hybridisation experiment using a hapten labelled oligonucleotide probe and a secondary antibody. (not to scale).

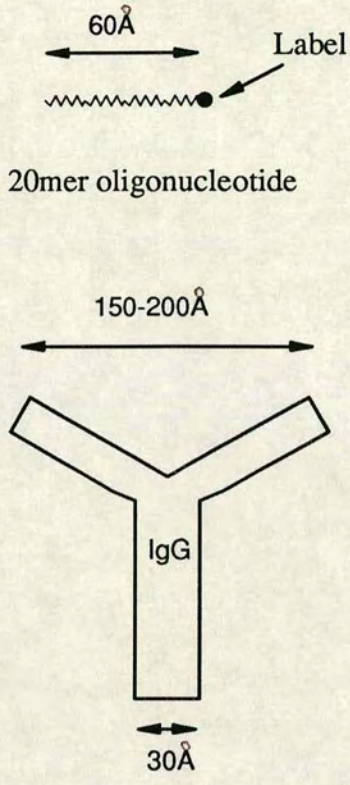


Figure 2b: Relative sizes of a 20mer oligonucleotide and an IgG antibody. (approximately to scale).

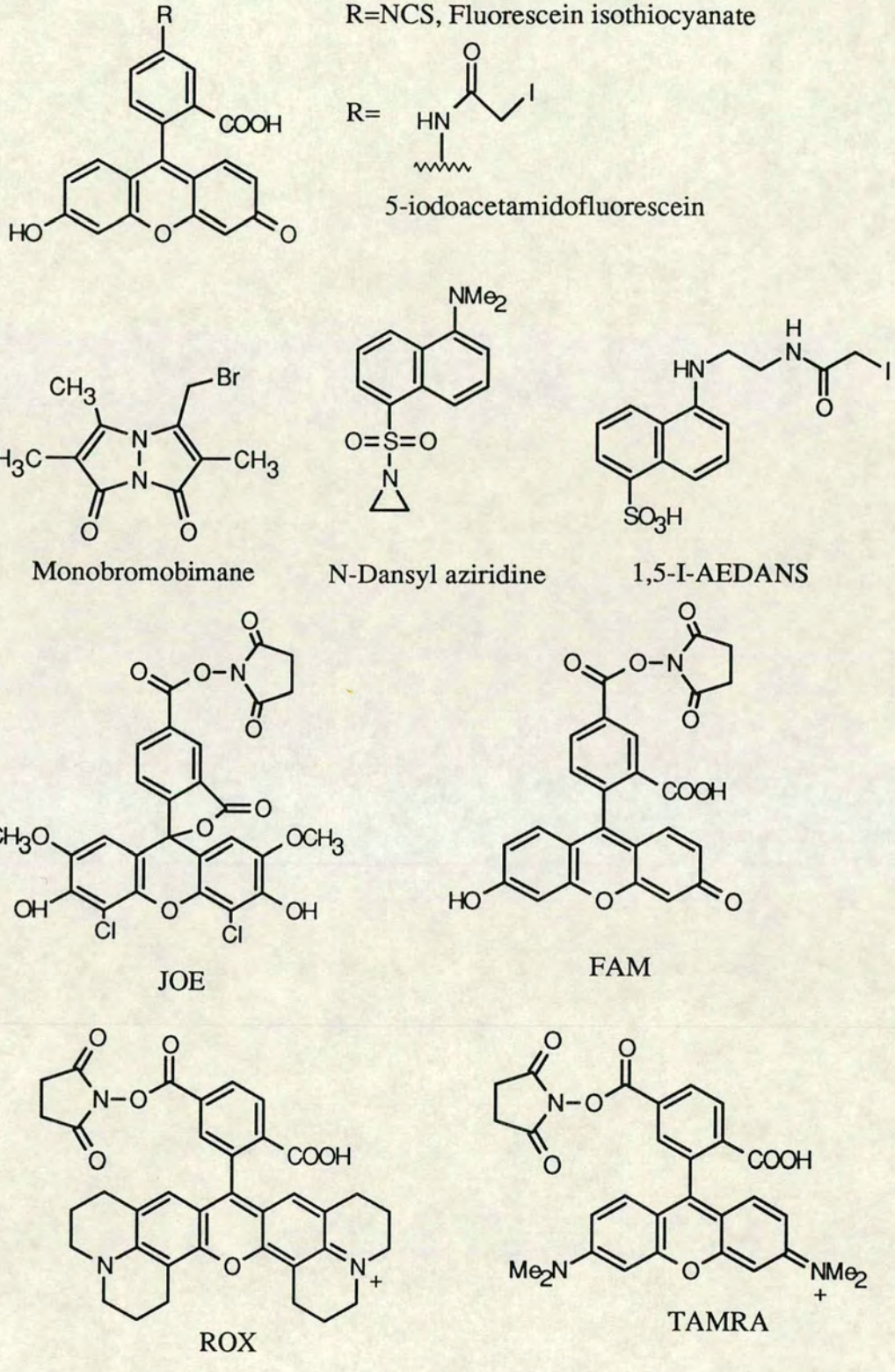


Figure 3: Amine- and Thiol-Reactive Derivatives of Fluorescent Dyes.

the dye iodoacetamide with a thiol-modified oligonucleotide (described in detail in section 3.1.2. of this chapter). Dyes can be chosen which fluoresce at different wavelengths, allowing simultaneous detection of different oligonucleotides labelled with different fluorophores. This is the basis of one type of automated DNA sequencing.[5-9] The use of a single fluorophore in automated DNA sequencing is also possible.[10] Fluorescent oligonucleotides are also used for *in situ* hybridisation experiments,[11] where fluorescence microscopy is used to detect the position of the hybridised fluorescent oligonucleotide. Cellular uptake of fluorescently labelled antisense oligonucleotides can be verified and quantified (described in Chapter 2 of this thesis).

2.1.2. *Enzymes*

Direct attachment of Alkaline phosphatase (AP) or Horseradish peroxidase (HRP) to oligonucleotides has been achieved by a variety of means.[12-15] Colorimetric detection of AP is usually carried out using a mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT), resulting in the production of a blue/purple precipitate.[16-19] A much wider choice of substrates is available for the colorimetric detection of HRP. Substrates which produce an insoluble coloured precipitate, such as 3,3'-diaminobenzidine (DAB), are of use where a semi-permanent record of the result is required, such as in *in situ* hybridization.[20] Substrates which produce a soluble coloured product are useful where quantitation of the signal is required as the colour can be measured spectrophotometrically.

Chemiluminescent detection has several advantages over colorimetric detection:

(i) The background is theoretically zero.

(ii) Detection is very rapid, typically involving a 20 second exposure to photographic film or a charge-coupled device.

(iii) Quantitation of the observed signal is relatively easy using a charge-coupled device, unlike colorimetric detection where quantitation by densitometer is very unreliable.

(iv) The labelled probe can be washed off, allowing several hybridisations to be carried out on the same filter-bound sample, unlike colorimetric detection where the coloured precipitate cannot normally be washed off.

The oxidation of 3-aminophthalhydrazide (luminol),[21-24] (*Figure 4*) by HRP in the presence of hydrogen peroxide results in chemiluminescence. The intensity of this chemiluminescence can be increased by the use of certain enhancers (*eg. para-* hydroxycinnamic acid or *para-* iodophenol).

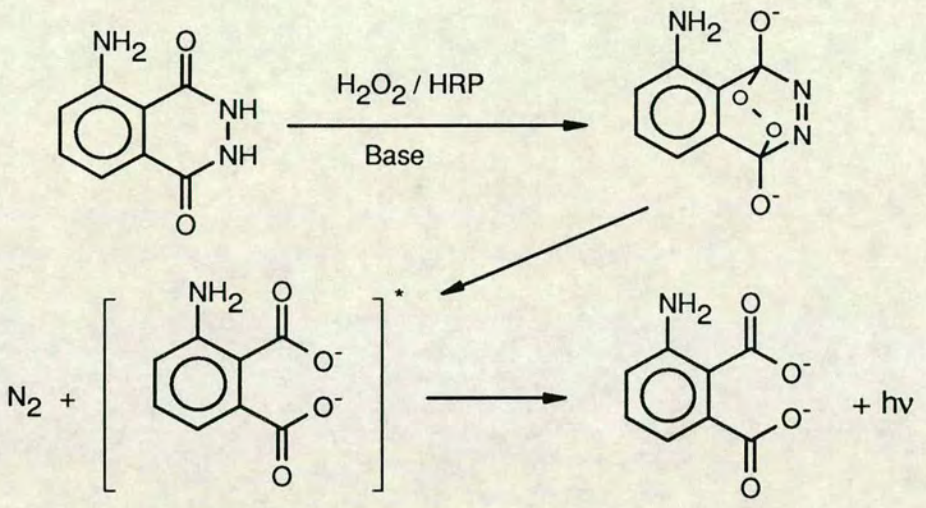
An alternative chemiluminescent reaction is that between the dioxetane produced by photooxygenation of 3-phosphate-9H-xanthen-9-ylideneadamantane, monopyridinium salt (AMPPD) and AP (*Figure 4*).[25]

This direct approach to labelling has the advantage of speed and convenience of detection. The disadvantages are:

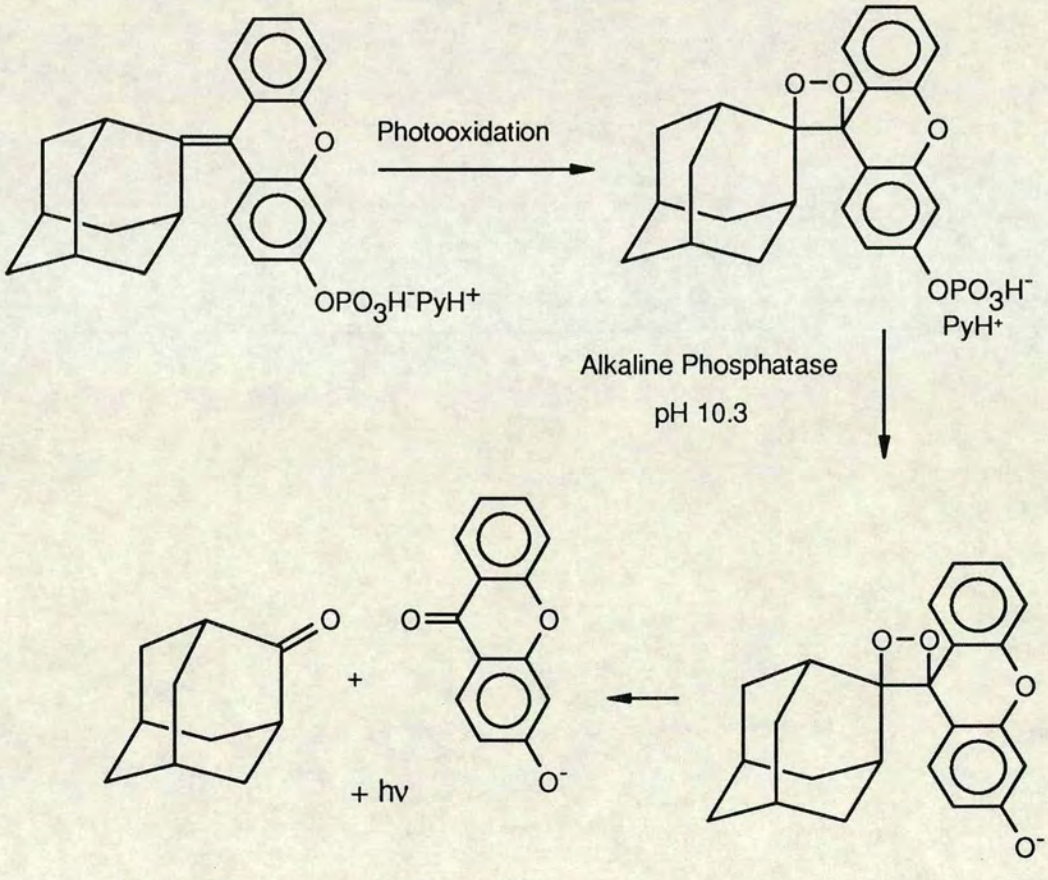
(i) The synthesis and purification of the enzyme-oligonucleotide conjugate is expensive and time consuming.

(ii) Only one enzyme can be attached per oligonucleotide

(iii) The activity of the enzyme may be impaired.



Possible mechanism of chemiluminescent oxidation of Luminol



Chemiluminescence from AMPPD

Figure 4: Chemiluminescent substrates for Horseradish Peroxidase and Alkaline Phosphatase

(iv)The hybridisation properties of the enzyme-labelled probe may be affected. Jablonski *et al.* found that the melting temperature of an AP-linked oligonucleotide was 10°C lower than that of an underivatized oligonucleotide.[12]

(v) HRP cannot be used in hybridisation experiments requiring incubation at >42°C, as it loses activity rapidly on heating above this temperature.[21]

2.2 *Indirectly Detectable Labelling Groups.*

2.2.1. *Biotin*

Biotin (*Figure 5*) forms a high affinity complex with the egg-white glycoprotein avidin and with the non-glycosylated bacterially-derived protein streptavidin both of which have four biotin binding sites.[25] The method of detection involves incubation of the biotinylated oligonucleotide with an avidin (or streptavidin)-enzyme conjugate. This results in the formation of an oligonucleotide-biotin-avidin-enzyme complex which is then incubated with the appropriate enzyme substrate to give a colorimetric or chemiluminescent signal. *Anti*-biotin antibodies can also be used for detection.[3,20,27] Although these antibodies have a lower affinity for biotin than (strept)avidin (K_d~10^{-12} M compared to 10^{-15}M for streptavidin), the use of a secondary antibody-enzyme conjugate allows enhancement in sensitivity. However, biotin is a very expensive hapten and much simpler and less expensive haptens could be used. The synthesis of derivatives of biotin is made difficult by its insolubility in most solvents. Biotin is unsuitable for use in certain *in situ* hybridization applications due to the high level of endogenous biotin found in some tissues and samples of biological origin.

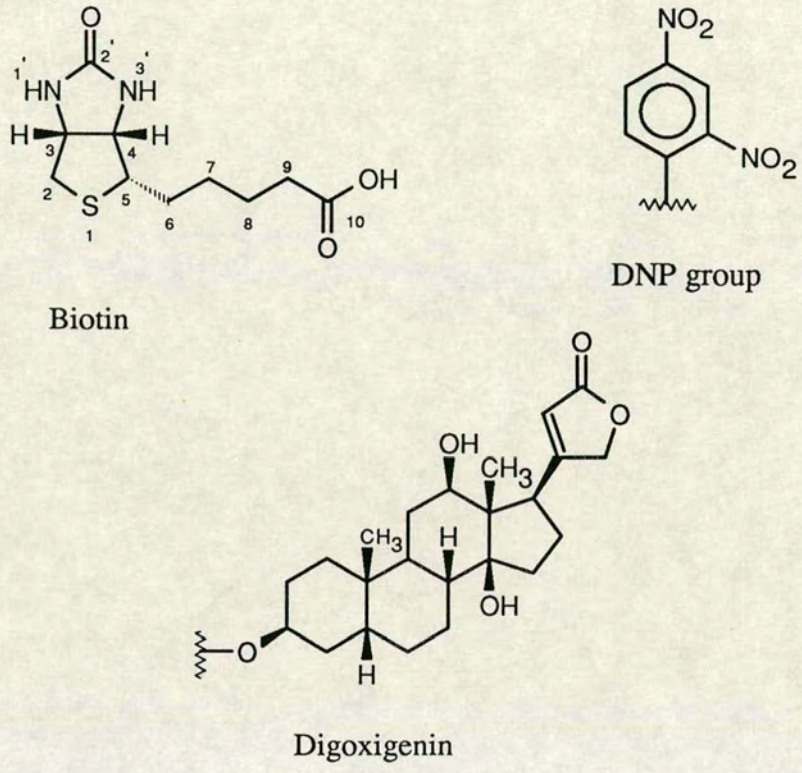


Figure 5: The three most common groups for indirectly detectable labelling of oligonucleotides

2.2.2. *Digoxigenin.*

Digoxigenin[28,29] (*Figure 5*) has become the leading alternative to biotin for use in *in situ* hybridization experiments where high levels of endogenous biotin cause high background signal problems. It does not occur naturally in clinical samples and therefore does not give rise to an undesirable background. It is detected immunogenically, using anti-digoxigenin polyclonal antibodies conjugated to AP. The sensitivity of detection of digoxigenin is comparable to that of biotin in DNA containing multiple digoxigenin groups. Digoxigenin is expensive, and the synthetic routes to its derivatives are complex due to the multifunctionality of the molecule.[4]

2.2.3. *The 2,4-Dinitrophenyl (DNP) Group.*

The 2,4-Dinitrophenyl (DNP) group (*Figure 5*) is an attractive labelling group for the following reasons:

(i) It is small. This minimises the possibility of interference with oligonucleotide hybridization.
(ii) It is inexpensive (unlike biotin and digoxigenin).
(iii) It is chemically simple (unlike digoxigenin).
(iv) It is extremely immunogenic. Very high affinity *anti*-DNP antibodies are commercially available from a number of sources.
(v) The DNP group is not found *in vivo*.

So far the practical and commercial applications of this labelling group have not been fully investigated. The methods used previously for introduction of the DNP group are:

(i) Reaction of 1-(2,4-dinitrophenylamino)-6-aminohexane with DNA which had been treated with N-bromosuccinimide. The bromine atoms on the modified bases generated in this way were displaced by the free amine resulting in a spaced DNP adduct.[30]
(ii) Enzymatic incorporation of 8-aminohexyl adenosine triphosphate follow by reaction of the amino-modified DNA with 2,4-dinitrofluorobenzene, or enzymatic incorporation of 8-(2,4-dinitrophenylamino)hexyl adenosine triphosphate.[31]
(iii) Photochemical attachment of a DNP-aryl azide.[32]

2.2.4. *N-Acetoxy-N^{2}acetyl amino fluorene.(AAAF).*

This group and it's 7-iodo derivative have been incorporated into DNA by direct reaction with the 8-position of purines and detected using an anti-AAAF antibody followed by a secondary antibody-AP conjugate.[33,34] The potential use of this labelling group is limited due to the carcinogenicity of AAAF and its derivatives.

2.2.5.*Phosphotyrosine.*

This group has been incorporated into oligonucleotides using a phosphotyrosine phosphoramidite.[27] It was detected using commercially available anti-phosphotyrosine antibody followed by a secondary antibody-HRP conjugate. The use of this label for nucleic acid detection has only been reported once, and its general applicability has yet to be demonstrated.

2.2.6.*Fluorescein.*

As well as its use as a fluorescent reporter group, fluorescein can also be detected using *anti*-fluorescein antibodies.[35]

3.0. Methods of introducing labelling groups into oligonucleotides.

3.1. *Post synthetically.*

Post synthetic methods of introducing non-radioactive labels into oligonucleotides are the most commonly used at present. They involve the synthesis and purification of the oligonucleotide to be labelled, followed by an enzymatic or chemical reaction to link the label to the oligonucleotide. The labelled oligonucleotide then has to be purified. The post-synthetic reactions are not suitable for automation, making the routine synthesis of labelled oligonucleotides a time-consuming task.

3.1.1.*Enzymatic Labelling of Oligonucleotides.*

Modified nucleoside triphosphates carrying the labelling group can be incorporated into oligonucleotides *via* enzymes such as deoxynucleotidyl terminal transferase which attaches a 'tail' of ribo- or deoxyribo-nucleotides at the 3'-end of oligonucleotides.[35] The most commonly used modified nucleoside triphosphates are the commercially available 5-(N-(N-biotinyl-ε-aminocaproyl)-3-amino-allyl) uridine 5'-triphosphate (Bio-11-UTP), and 5-((N-biotinyl)-3-amino-allyl) uridine 5'-triphosphate (Bio-4-UTP) and their 2'-deoxyuridine analogues[3,35-37] (*Figure 6*). Biotinylated derivatives of 8-aminohexyl adenosine 5'-triphosphate are also commercially available. The DNP derivative of 8-aminohexyl adenosine 5'-triphosphate has been successfully incorporated into DNA.[31] Digoxigenin can be enzymatically incorporated into DNA using Digoxigenin-O-succinyl-ε-aminocaproyl-[5-(3-aminoallyl)-2'-deoxy uridine-5'-triphosphate] (Dig-11-dUTP), and Digoxigenin-O-succinyl-[5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate] (Dig-4-dUTP)[4,29] (*Figure 6*).

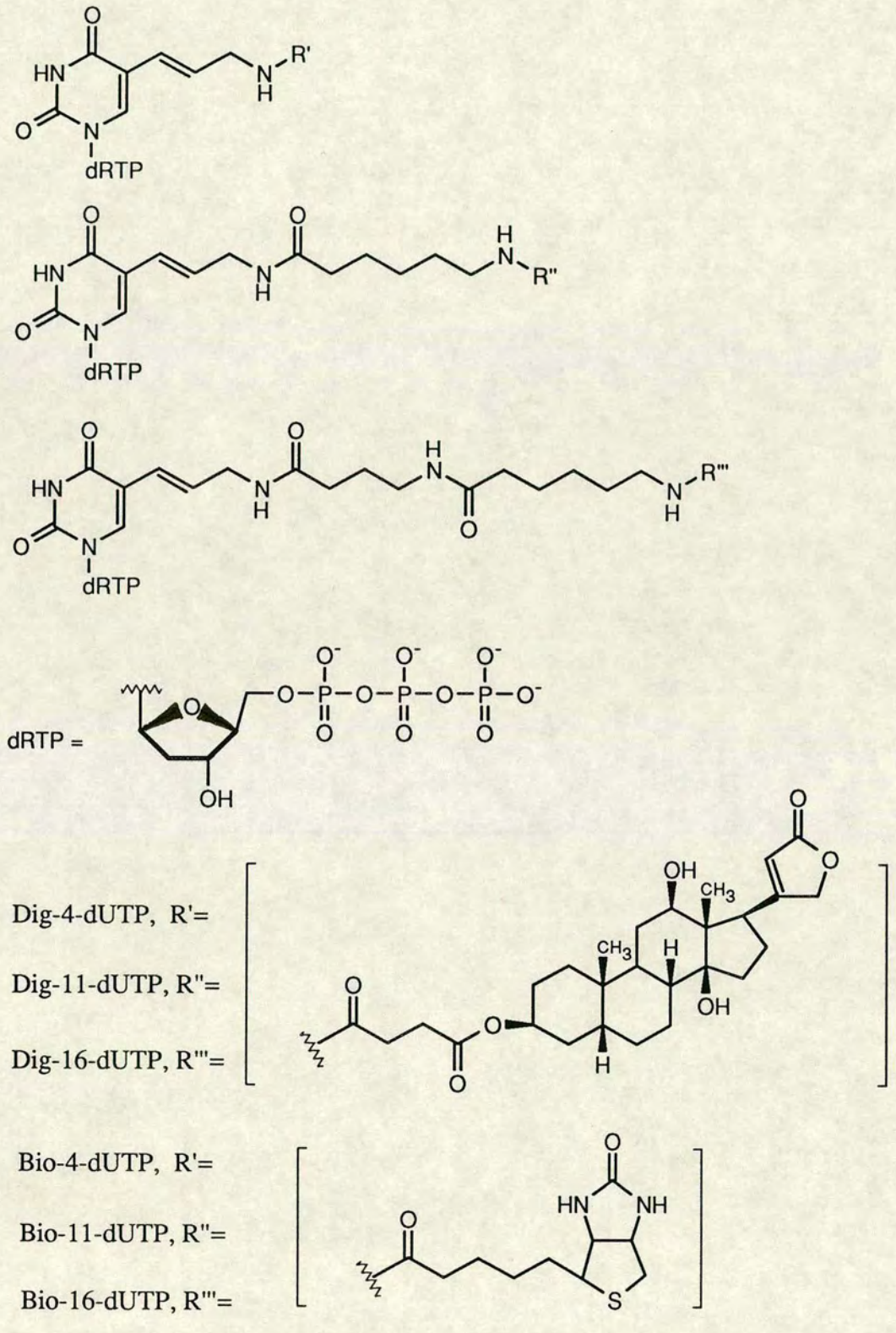


Figure 6: Nucleoside triphosphates for enzymatic incorporation of Biotin and Digoxigenin

Cosstick *et al.* introduced a single 2'-deoxycytidine-5'-phosphate-3'-thiophosphate coupled to bimane *via* the thiophosphate into RNA by the action of T4 RNA ligase.[38]

Other enzymatic methods of introducing modified bases into DNA, such as nick translation and random priming are not generally applicable to synthetic oligonucleotides and are described in detail by Keller and Manak.[2]

The enzymatic labelling of oligonucleotides is not feasible on a large scale and is expensive and time-consuming.

3.1.2. *Chemical Labelling of Oligonucleotides.*

Post synthetic chemical labelling of oligonucleotides was, until recently, the most widely used technique for the attachment of reporter groups to oligonucleotides, and for some labelling groups it is still the only method available. The general approach is the attachment of a reactive group (usually a nucleophile such as an aliphatic amine or thiol) to the oligonucleotide either during or after solid-phase synthesis, and subsequent reaction of the reactive group with an activated labelling molecule.[39]

3.1.2.1. *Amino-modification of oligonucleotides.*

The most common method of chemical labelling is the attachment of a linker molecule containing a primary aliphatic amino function to the oligonucleotide, followed by reaction with an amino-reactive labelling molecule, such as an active ester or isothiocyanate, or with an enzyme. Urdea *et. al.* have reported a variety of useful protocols for the attachment of amine reactive labels to amino-modified oligonucleotides.[15]

The standard method of introducing a primary amino moiety onto the 5'-end of oligonucleotides is *via* commercially available 'AMINOLINK 2' (*Figure 7a*).[40] This is the 2-cyanoethyl phosphoramidite of 1-trifluoroacetylaminohexan-6-ol. This can be reacted with a huge variety of amine-reactive labelling groups such as fluorescein isothiocyanate (FITC), active esters and isothiocyanates of other fluorophores, biotin-N-hydroxysuccinimide ester, and digoxigenin N-hydroxysuccinimide ester.[41] A large number of very similar compounds based on $H_2N(CH_2)_nOH$ has also been synthesized, including:

-The methyl phosphoramidite of N-(monomethoxytrityl)-3-amino propan-1-ol was prepared and used by Connolly[42] to synthesize biotinylated and dansylated oligonucleotides.

-Tanaka *et al.* synthesized a phosphotriester monomer of N-(monomethoxytrityl)-2-amino ethanol.[43]

-Coull *et al.* synthesized the 2-cyanoethyl phosphoramidite of N-(trifluoroacetyl)-1-amino ethanol.[44]

-Agrawal *et al.* synthesized the 2-cyanoethyl phosphoramidite of N-Fmoc-aminoethanol.[45]

-Kaiser *et al.* synthesized N-Fmoc protected aminoethanol and 6-amino-hexan-1-ol phosphoramidites.[8]

-Sinha and Cook synthesized phosphoramidites and hydrogen-phosphonate monomers of N-trityl 6-amino-hexan-1-ol.[46]

-Bannwarth *et al.* synthesized the 2-cyanoethyl phosphoramidite of N-(monomethoxytrityl)-aminopropan-3-ol which was reacted with the NHS active ester of a fluorescent bathophenanthroline-ruthenium (II) complex.[47]

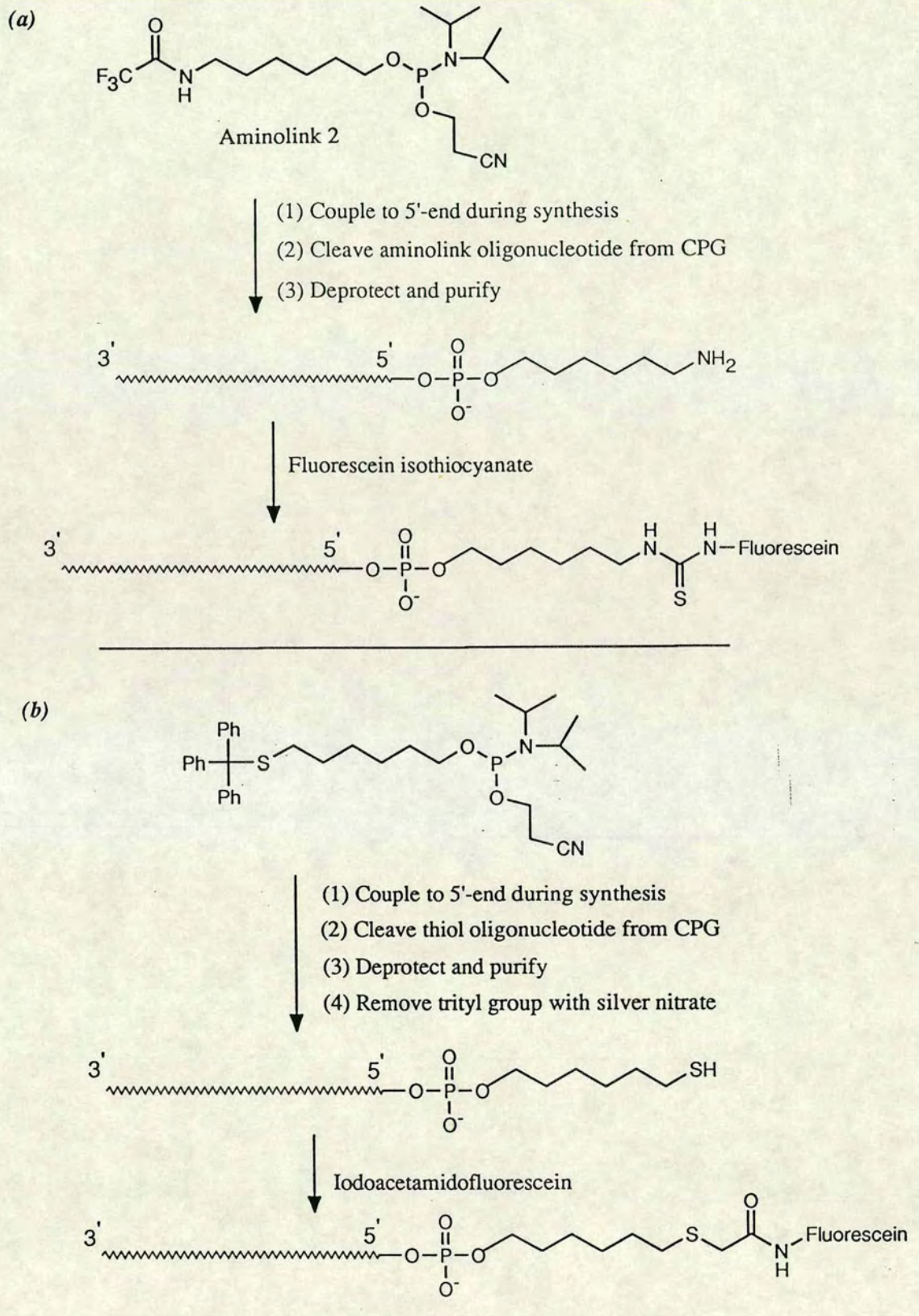


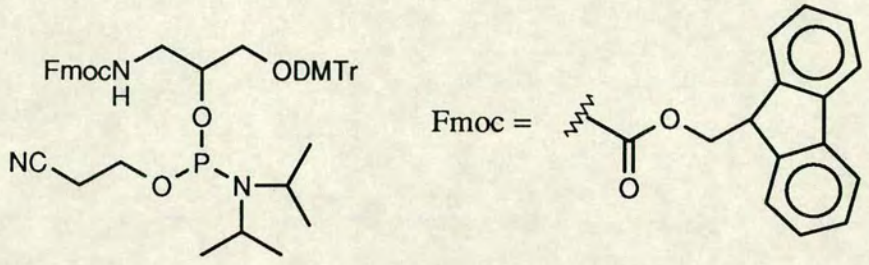
Figure 7: Chemical fluoresceination of (a) aminomodified and (b) thiol modified oligonucleotides

-Agrawal synthesized a methylphosphonate monomer of N-Fmoc-6-amino hexan-1-ol. This was to be used in the study of cellular uptake of methylphosphonate oligonucleotides.[48]

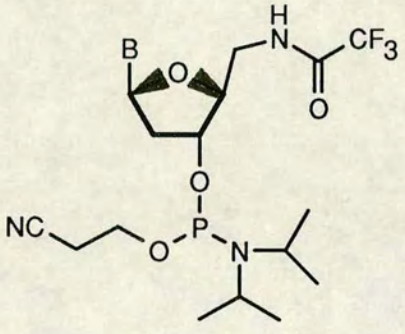
Multiple amino functions have been incorporated into oligonucleotides using a simple 1-amino-propane diol based phosphoramidite (*Figure 8*). [49] This was used to synthesize an oligonucleotide probe containing up to five biotins, however no comparison between the sensitivity of detection of this probe and a singly biotinylated probe is reported. This backbone was also used to produce an amino-functionalized controlled-pore glass for the synthesis of 3'-aminomodified oligonucleotides.[50]

Smith *et al.* used a protected 5'-amino-5'-deoxythymidine phosphoramidite to introduce a single amino function at the 5'-end of oligonucleotides.[5] This was then used to produce sequencing primers labelled with four spectrally resolved fluorophores. These were to be used in automated DNA sequencing. An analogous approach was used by Sproat *et al.* to synthesize all four 5'-amino-2',5'-dideoxynucleoside phosphoramidites for attachment of Iridium complexes and other labels to oligonucleotides[51] (*Figure 8*). Manoharan *et al.* incorporated multiple amino functions into oligonucleotides during solid-phase synthesis by means of a protected 2'-O-alkylaminoribonucleoside phosphoramidite.[52] These were then used to attach a variety of labelling groups to oligonucleotides, as well as cholic acid in an attempt to enhance cellular uptake of 'antisense' oligonucleotides.

More involved methods of introducing aliphatic amines into oligonucleotides include:

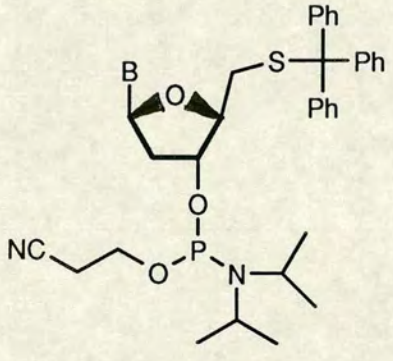


Multiple Addition Aminomodifier of Nelson et. al. (ref. 49)



B=thymine
N4-benzoyl cytosine
N2-isobutyryl guanine
N6-pivaloyl adenine

5'-amino-2',5'-dideoxynucleoside phosphoramidites of Sproat et. al. (ref. 51)



B=thymine
N4-benzoyl cytosine
N2-isobutyryl guanine
N6-pivaloyl adenine

5'-mercapto-2',5'-dideoxynucleoside phosphoramidites of Sproat et. al. (ref. 75)

Figure 8: Amino- and Thiol- modifier Phosphoramidites

-the carbodiimide coupling of a protected aminoalkyl phosphonate to the 5'-hydroxyl group of oligonucleotides was carried out by Kansal *et al.* [53]

-the formation of 5'-phosphorimidazolide of an oligonucleotide by a carbodiimide coupling, followed by reaction with an alkyl diamine to form a 5'-aminoalkyl phosphoramidate.[26,54]

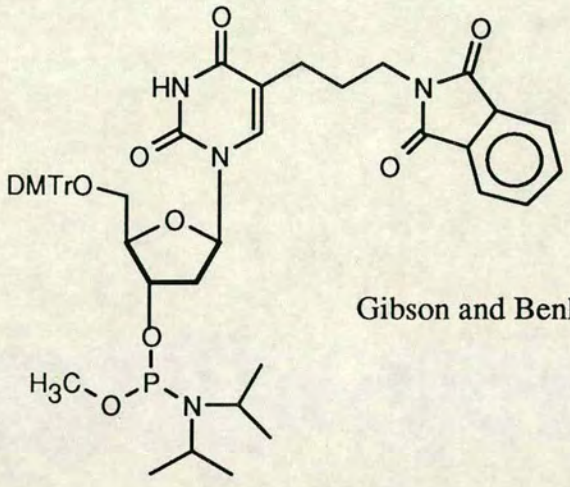
-oxidative amination of a hydrogen-phosphonate internucleoside linkage with N-1-(trifluoroacetyl)-hexane diamine.[55-57]

-reaction of the 5'-hydroxyl group with the bifunctional reagent carbonyldi-imidazole (a phosgene equivalent) followed by reaction with hexane diamine gave a carbamate-linked amine. The carbamate linkage was found to be stable to ammonia deprotection conditions.[58]

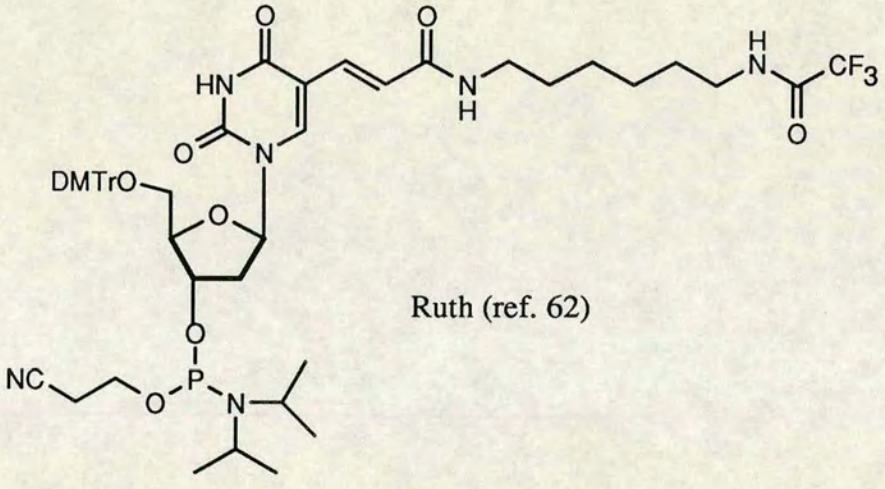
The solid-phase synthesis of oligonucleotide-peptide conjugates on a controlled-pore glass solid support has been carried out by Haralambidis *et. al.* [59,60] Oligonucleotides labelled with various numbers of lysine residues were labelled with multiple biotin and fluorophore residues. This approach to labelling requires the use of both a peptide synthesizer and a DNA synthesizer, and is thus not of practical use for non-radioactive labelling.

In spite of its expense the introduction of primary amino functions into oligonucleotides *via* modified nucleosides has been used quite frequently. Such modified nucleosides include of C-5 aminoalkyl or aminoallyl 2'-deoxyuridine derivatives (*Figure 9*), [61-64] N-4 aminoalkyl deoxycytidine derivatives (*Figure 9*), [15,65,66] C-8 aminoalkyl purines[31,35] and N6-aminoalkyl (2'-deoxy)adenosine.[35]

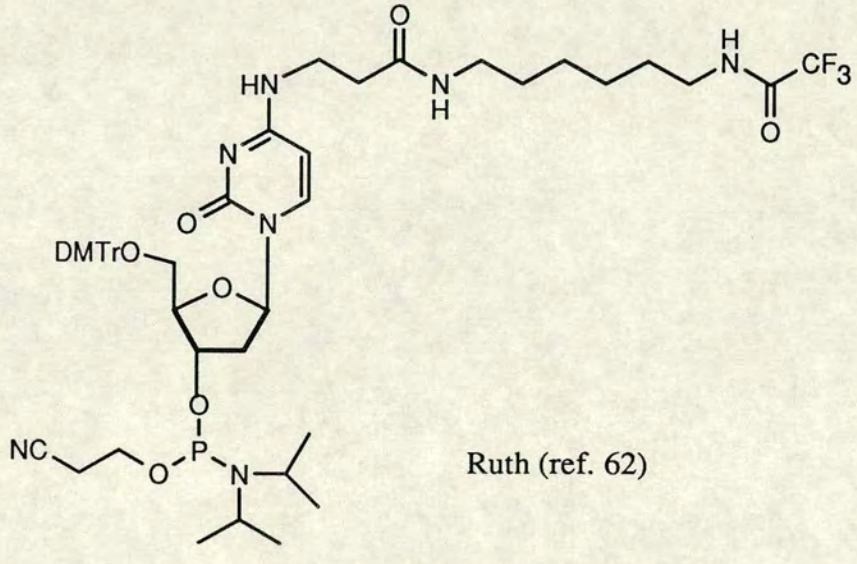
One advantage of the use of 5'-non-isotopically labelled nucleotides is that it is possible to introduce 5'-radiolabels onto the oligonucleotide using polynucleotide kinase. However the efficiency of



Gibson and Benkovic (ref. 63)



Ruth (ref. 62)



Ruth (ref. 62)

Figure 9: Modified Base Amino-modifier Phosphoramidites

enzymic radiolabelling is lower in the presence of the modified 5'-terminal nucleotides.[66]

The modified nucleoside approach to the introduction of primary amino functions allows the multiple internal labelling of oligonucleotides as well as end labelling. However the sensitivity of detection of internal biotin labels has been shown to be less than that of biotin attached at the 3'- or 5'- end of oligonucleotides.[64]

The effect on oligonucleotide hybridisation of the modifications to the bases is also a problem.[64,67,68] Telser *et. al.* showed that oligonucleotides containing N-4 aminoalkyl substituted deoxycytidine

derivatives gave biphasic melting curves, and those containing C-5 aminoalkyl 2'-deoxyuridine derivatives were destabilised relative to unmodified oligonucleotides.[67] In addition, this approach to labelling is expensive, mainly due to the cost of the deoxynucleotide precursors.

3.1.2.2. *Thiol-modified oligonucleotides.*

The next most common method of chemical labelling is the attachment of a thiol group, a thiophosphate or a phosphorothioate-diester internucleoside linkage to the oligonucleotide, followed by reaction with sulphur-reactive labelling molecules, such as maleimides, iodo(or bromo)acetamides, γ-bromo-α,β-unsaturated carbonyls, and aziridinyl sulphonamides (*Figure 3*), or with disulphide-containing or bromoacetylated enzymes.

The attachment of thiols to the 3'-end of oligonucleotides has been achieved during solid-phase synthesis using functionalised solid supports.[69-72] In all these cases the thiol was protected as a disulphide

during oligonucleotide synthesis. After synthesis and deprotection the disulphide was cleaved when required using dithiothreitol. Bonfils and Thuong[69] synthesized oligonucleotides containing a 3'-disulphide-protected thiol and a 5'-thiophosphate. This allowed the labelling of the 5'-thiophosphate using 5-iodoacetamidofluorescein (*Figure 3*). Subsequent treatment of the fluorescent oligonucleotide with dithiothreitol gave a free thiol to be used to conjugate the oligonucleotide to proteins. Gupta *et al.* [70] synthesized fluorescent oligonucleotides by reacting 5-(2-(iodoacetyl)aminoethyl) aminonaphthalene-1-sulphonic acid (1,5-I-AEDANS) (*Figure 3*) with the 3'-thiol generated using their disulphide solid-support. Li *et al.* [71] synthesized an N-4-aminoethyl-2'-deoxycytidine derivatized solid support. This was used to synthesize 3'-amino-modified oligonucleotides. On reaction with dithio-bis-propionyl-N-hydroxy succinimide and treatment with DTT these were converted to 3'-thiol modified oligonucleotides which were conjugated to alkaline phosphatase which had been bromoacetylated using bromoacetic acid N-hydroxysuccinimide ester. Zuckermann *et al.* [72] used 3'-derivatised thymidine on a solid support to form 3'-thiol-containing oligonucleotides. Enzymatic tailing at the 3'-end with 4-thio-2'-deoxyuridine and reaction with sulphur-reactive fluorophores has been carried out by Eshaghpour *et al.* [73]

The synthesis of 5'-thiol modified oligonucleotides has been carried out using a protected thiol phosphoramidite (*Figure 7b*)[74] and hydrogen phosphonate monomers[46] based on S-trityl-mercaptoalkanols. The trityl group was removed after synthesis using silver ions to give the free thiol which on reaction with biotin maleimide or a variety of sulphur-reactive fluorophores gave labelled oligonucleotides. Saiki *et al.* used the 2-cyanoethyl phosphoramidite of an S-trityl mercaptan derivative made

from tetraethylene glycol to introduce a free thiol onto the 5'-end of oligonucleotide probes.[14] The thiol was then reacted with maleimido derivatised HRP to give HRP labelled oligonucleotides. Sproat *et al.* synthesized all four base protected 5'-(S-trityl)mercapto-2',5'-dideoxyribonucleoside 3'-O-(2-cyanoethyl) N,N-diisopropyl phosphoramidites[75] (*Figure 8*). The 5'-thiol generated on treatment of the oligonucleotide with silver nitrate was reacted with 5-iodoacetamidofluorescein to generate fluorescently labelled oligonucleotides. Kumar *et al.* synthesized 5'-thiol modified oligonucleotides by reaction of a 5'-*amino modified* oligonucleotide with N-acetyl-DL-homocysteine-thiolactone.[76] The resulting thiol oligonucleotides were fluorescently labelled with N-(3-pyrenyl)-maleimide. A similar approach was used by Gaur *et al.*, [77] who reacted N-succinimidyl-3-(2-pyridyldithio)-propionate with a 5'-amino modified oligonucleotide. Subsequent treatment with DTT liberated the thiol, as confirmed by the release of pyridine-2-thione. Murakami *et al.* [78] synthesized AP-oligonucleotide conjugates by the carbodiimide coupling of a 5'-phosphate oligonucleotide to cystamine. Cleavage of the resulting 5'-disulphide with DTT and reaction of the thiol with disulphide-modified AP gave the desired product. This approach was first used by Chu and Orgel to synthesize oligonucleotides conjugated to a number of proteins, including peroxidase.[54]

The chemical reactivity of phosphorothioate-diester-containing oligonucleotides has been exploited by Fidanza and McLaughlin[79] to give 'backbone labelled' oligonucleotides. The number and position of the phosphorothioate moieties can be varied to allow the incorporation of multiple labelling groups. Phosphorothioate oligonucleotides were labelled with the PROXYL spin-label *via* its iodoacetamide; with dansyl

groups by reaction with N-dansyl aziridine or 1,5-I-AEDANS (*Figure 3*) and with a γ-bromo-α,β-unsaturated carbonyl-based drug molecule.[80] Agrawal and Zamecnik[56] synthesized oligonucleotides containing two *different* labels by the use of oligonucleotides containing both a phosphorothioate-diester internucleoside link and an aminoalkyl phosphoramidate link. Successive reaction of the modified oligonucleotide with a sulphur-reactive labelling molecule, such as monobromobimane (*Figure 3*), then with an amine-reactive label, such as FITC or biotin-NHS ester, resulted in heterobifunctionally labelled oligonucleotides. 3'-Thiophosphate residues have been incorporated into RNA by the action of RNA ligase, and reacted with monobromobimane to produce fluorescently labelled RNA.[38]

3.1.2.3. *Attachment of a 5'-(5'-linked ribonucleotide) to oligonucleotides*

This novel approach was carried out by Agrawal *et al.*, who introduced a single 5'-(5'-linked ribonucleotide) into oligonucleotides during solid-phase synthesis[45] (*Figure 10*). The resulting 2'-3'-*cis* -diol was oxidized to the dialdehyde by sodium periodate. Reaction with biotin hydrazide, followed by reduction with sodium borohydride resulted in the attachment of a 5'-biotin moiety to the oligonucleotide.

All the procedures described above suffer from the incompatibility between the solubility of the labelling molecule and the oligonucleotide. This often results in low yield of the labelled oligonucleotide. In addition, the modifications to standard oligonucleotide synthesis and purification procedures,[81] as well as the post synthetic manipulations involved in the labelling reactions, add considerably to the time and cost of routine oligonucleotide labelling.

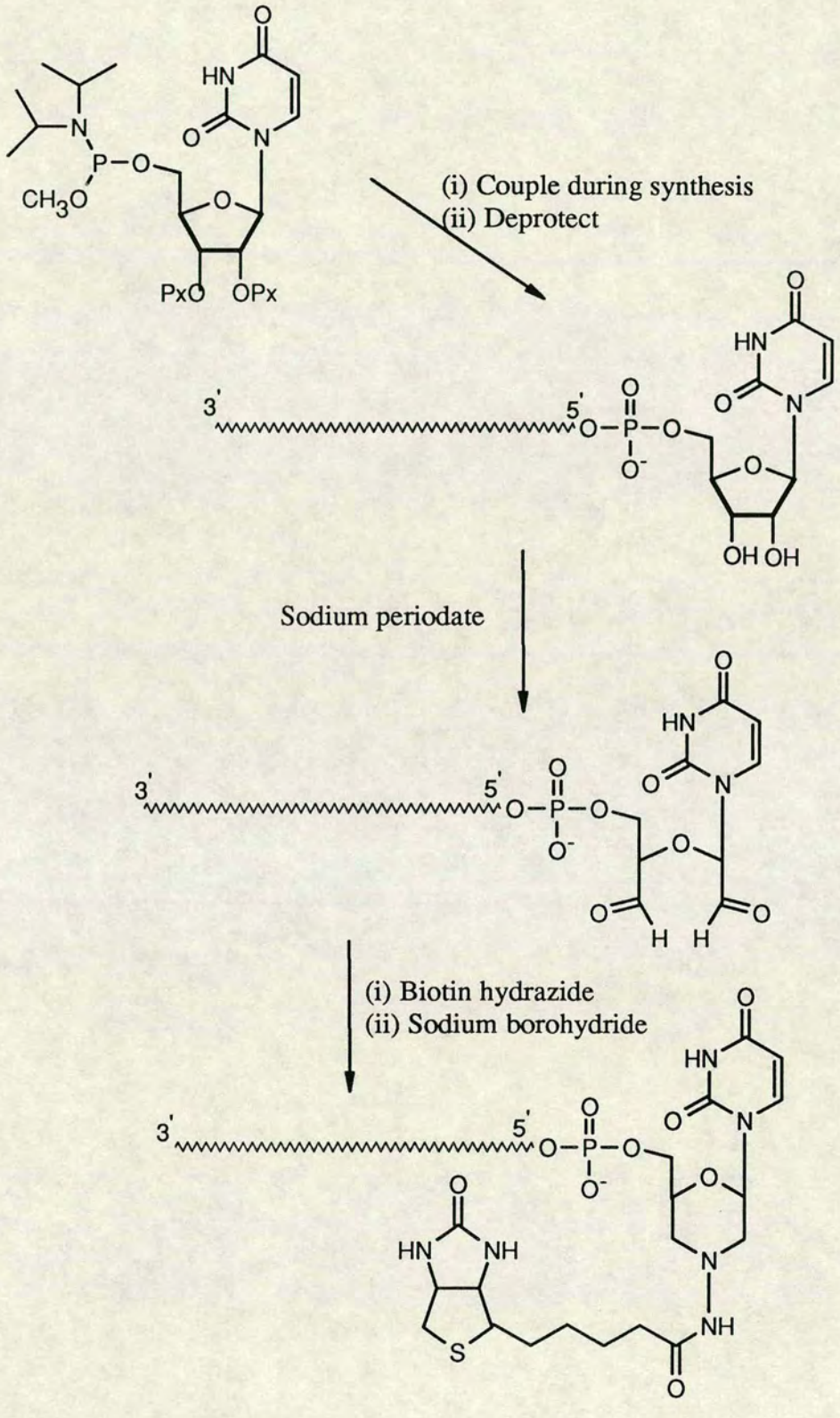
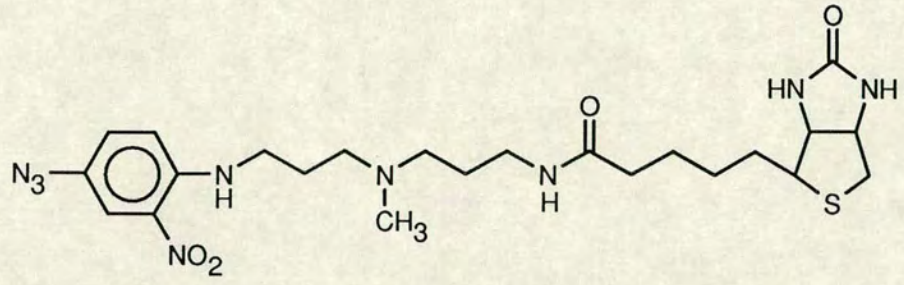


Figure 10: Biotinylation using the 5'-Uridine phosphoramidite of Agrawal et. al. (ref. 45)



Photobiotin

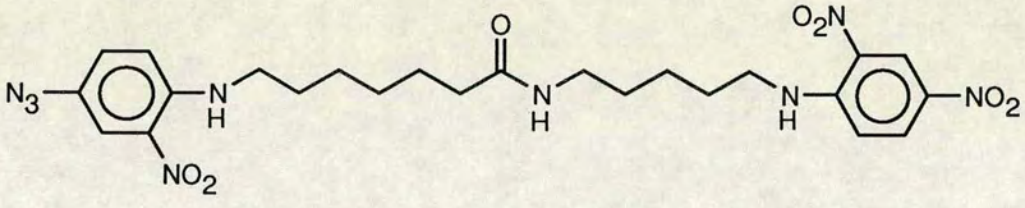


Photo-DNP

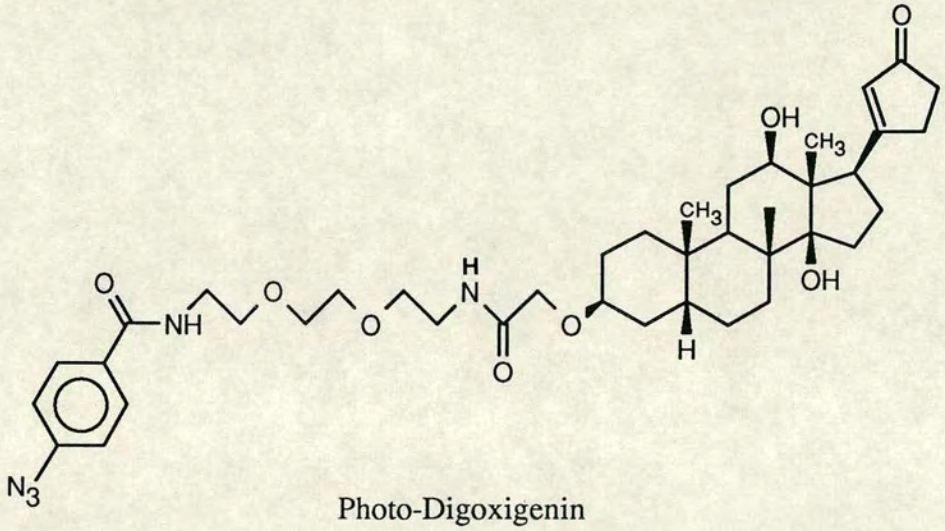


Photo-Digoxigenin

Figure 11: Photoactivatable Labelling Groups

3.1.3. *Photochemical Labelling of Oligonucleotides*

Another post-synthetic method of introducing labels into an oligonucleotide is *via* photoactivatable aryl azides. UV irradiation of a mixture of the label covalently linked to an aryl azide and the oligonucleotide results in the formation of a nitrene which reacts with the heterocyclic bases thereby attaching the labelling group to the oligonucleotide. The major disadvantage of this method is that the attachment of such a bulky group to the bases of the DNA in an uncontrolled manner is likely to inhibit hybridisation. Biotin can be attached to DNA using 'Photobiotin'[82], digoxigenin by 'Photodigoxigenin'[4] and the DNP group by 'Photo-DNP'[32] (*Figure 11*)

There is one report of a similar, but non-photochemical method of labelling oligonucleotides by the action of a diazonium salt of a biotin derivative.[83]

3.2. *Introduction of Labels during Solid-Phase Oligonucleotide Synthesis.*

The incorporation of the labelling group during the synthesis of the oligonucleotide is the quickest and most convenient way of introducing a non-radioactive label. After the synthesis is complete the labelled oligonucleotide is cleaved from the solid support, deprotected and purified. Another advantage of this approach is that the HPLC and gel electrophoretic properties of the labelled oligonucleotide are likely to be very different from those of unlabelled failure sequences, making purification relatively easy. This approach also makes it possible to attach multiple labels to the oligonucleotide in a controlled manner, in order to increase the signal and thus improve the sensitivity of detection.

Multiple labels have been introduced to the 3'- and 5'-OH end of oligonucleotides, and single labels have been introduced to the 5'-OH end.

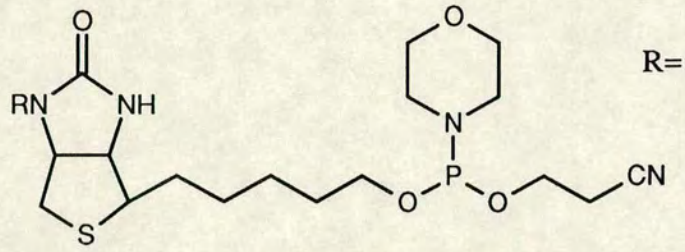
3.2.1. *Single Labelling*

The 5'-OH end of oligonucleotides has been successfully labelled with a single biotin group during solid-phase synthesis using the biotin phosphoramidite monomers described below. (*Figure 12*).

Alves *et al.* [85] synthesized methyl-morpholino biotin phosphoramidites by reduction of the methyl ester of N-1' dimethoxytrityl and N-1'-tetrahydropyranyl biotin. Phosphitylation of the resulting N-1'-protected biotinol gave the phosphoramidite.(*Figure 12*). The insolubility of biotin derivatives in organic solvents was overcome by the protection of N-1' of the ureido ring of biotin to increase the lipophilicity of the compound. The yield for the protection reactions were, however, only ~40%, making this a rather expensive method of producing a phosphoramidite monomer for labelling. In addition the phosphoramidites were insoluble in acetonitrile.

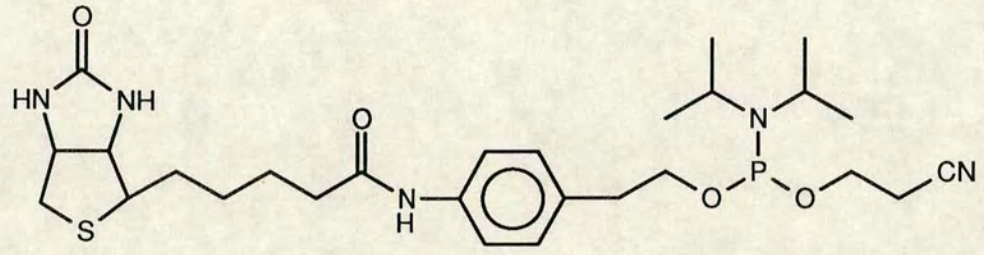
Cocuzza[86] synthesized a 2-cyanoethyl-diisopropylphosphoramidite of biotin in two steps by reaction of biotin N-hydroxysuccinimide ester with *p*-aminophenethyl alcohol followed by phosphitylation. The resulting phosphoramidite (*Figure 12*) is not protected at N-1', and was found to be insoluble in acetonitrile. In addition, the presence of an aromatic amide in the linker arm necessitated care in the ammonia deprotection step of oligonucleotide synthesis.

Building on the above work, Pon[87] synthesized the biotin phosphoramidite which has become the standard for introduction of biotin

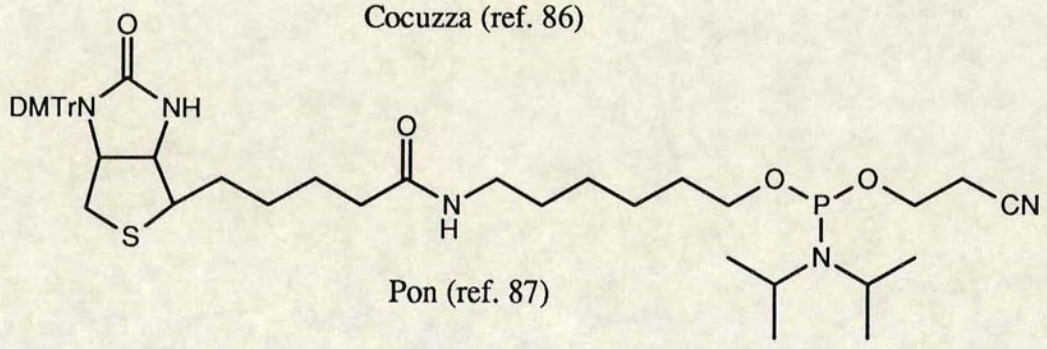


R= 4,4'-Dimethoxytrityl
or Tetrahydropyranyl

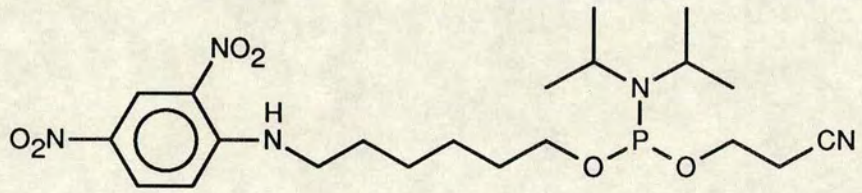
Alves et al. (ref 85)



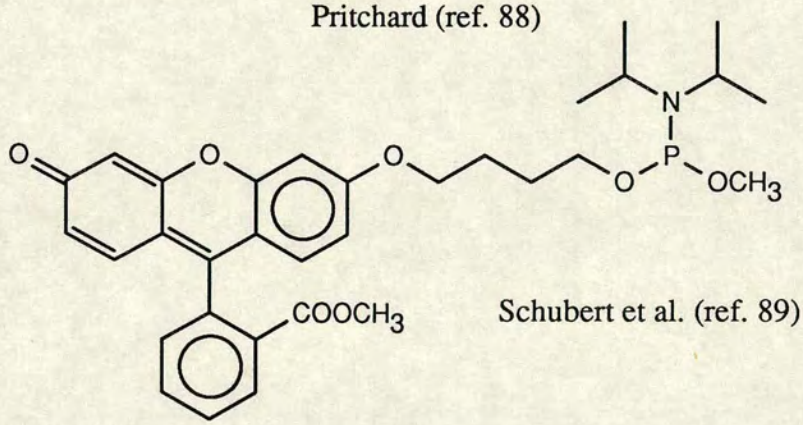
Cocuzza (ref. 86)



Pon (ref. 87)



Pritchard (ref. 88)



Schubert et al. (ref. 89)

Figure 12: Single addition labelling phosphoramidites

to oligonucleotides(*Figure 12*). Biotin was attached to 6-amino-1-*tert* - butyldimethylsilylhexan-1-ol using a carbodiimide coupling reaction. The resulting compound was reacted with 4,4'-dimethoxytrityl chloride to give the N-1' dimethoxytrityl analogue. The silyl ether was cleaved with TBAF, and subsequent phosphitylation gave the phosphoramidite. This phosphoramidite was soluble in acetonitrile, and was used under standard oligonucleotide synthesis conditions. The presence of the dimethoxytrityl group allows the coupling efficiency to be monitored. This was found to be around 95%, considerably higher than yields obtained by post-synthetic biotinylation methods described above. This phosphoramidite is commercially available (Cambridge Research Biochemicals).

The 5'-OH end of oligonucleotides has been labelled with a single DNP group using a 2-cyanoethyl-diisopropyl phosphoramidite monomer synthesized in this group[88] (*Figure 12*). The monomer was soluble in acetonitrile, and standard DNA synthesis conditions were used. Coupling efficiences were estimated by HPLC to be >97%. The label was found to be stable to ammonia deprotection conditions. Ultraviolet-melting studies showed that the attachment of this label had no effect on oligonucleotide hybridisation.

A simple fluorescein phosphoramidite has been synthesized by Schubert *et al.* [89] and used for the fluorescent labelling of oligonucleotides during solid-phase synthesis (*Figure 12*).

A phosphoramidite of a Bathophenanthroline-Ruthenium (II) complex has been successfully used by Bannwarth and Schmidt to fluorescently label oligonucleotides.[90] The complex was used as solution in acetonitrile with a 10 minute coupling time. The complex was found to be stable to DNA synthesis conditions.

3.2.2. *Multiple Labelling.*

Multiple labelling of oligonucleotides with a variety of groups has been achieved by Roget *et al.* [91] using a modified nucleoside approach.(*Figure 13a*). Dansyl, pyrenyl, biotinyl and dinitrophenyl derivatives of N-4 aminohexyl deoxycytidine derivatives have been synthesized and converted to 2-cyanoethyl-diisopropyl phosphoramidites. These were then used in the synthesis of oligonucleotides and gave coupling efficiencies of >97%. The DNP-labelled oligonucleotides were found to be unstable to the normal base deprotection conditions (5 hours at 55°C in NH_4OH), liberating 2,4-dinitrophenol. This necessitated the use of more base-labile base protecting groups on the DNA synthesis monomers.[92] The biotin- and dansyl-labelled oligonucleotides were found to be stable to normal base deprotection conditions. The biotinyl moiety was unprotected and this probably limits the use of this phosphoramidite to the introduction of biotin labels at the 5'-end of oligonucleotides as side reactions at N-1' are likely to occur on exposure to repeated oligonucleotide synthesis cycles.[93]

A similar approach has been used by Pieles *et al.* [93] to introduce multiple biotin labels into 2'-O-methyl- and 2'-O-allyl-oligo*ribo* nucleotides for use in the capture of RNA binding proteins (*Figure 13b*). The N-1' of the biotin is protected by a 4-*tert*-butyl benzoyl group. This allowed the synthesis of oligo*ribo*nucleotides biotinylated at the 3'-end. Attempts to synthesize the same sequences using an unprotected biotin phosphoramidite resulted in substantial side reactions and chain branching. This side-reaction problem is much worse for oligo*ribo*nucleotide synthesis than for standard oligo*deoxyribo*nucleotide synthesis due to the extended coupling time and the more efficient activator (5-(4-nitrophenyl)-1H-tetrazole) used.

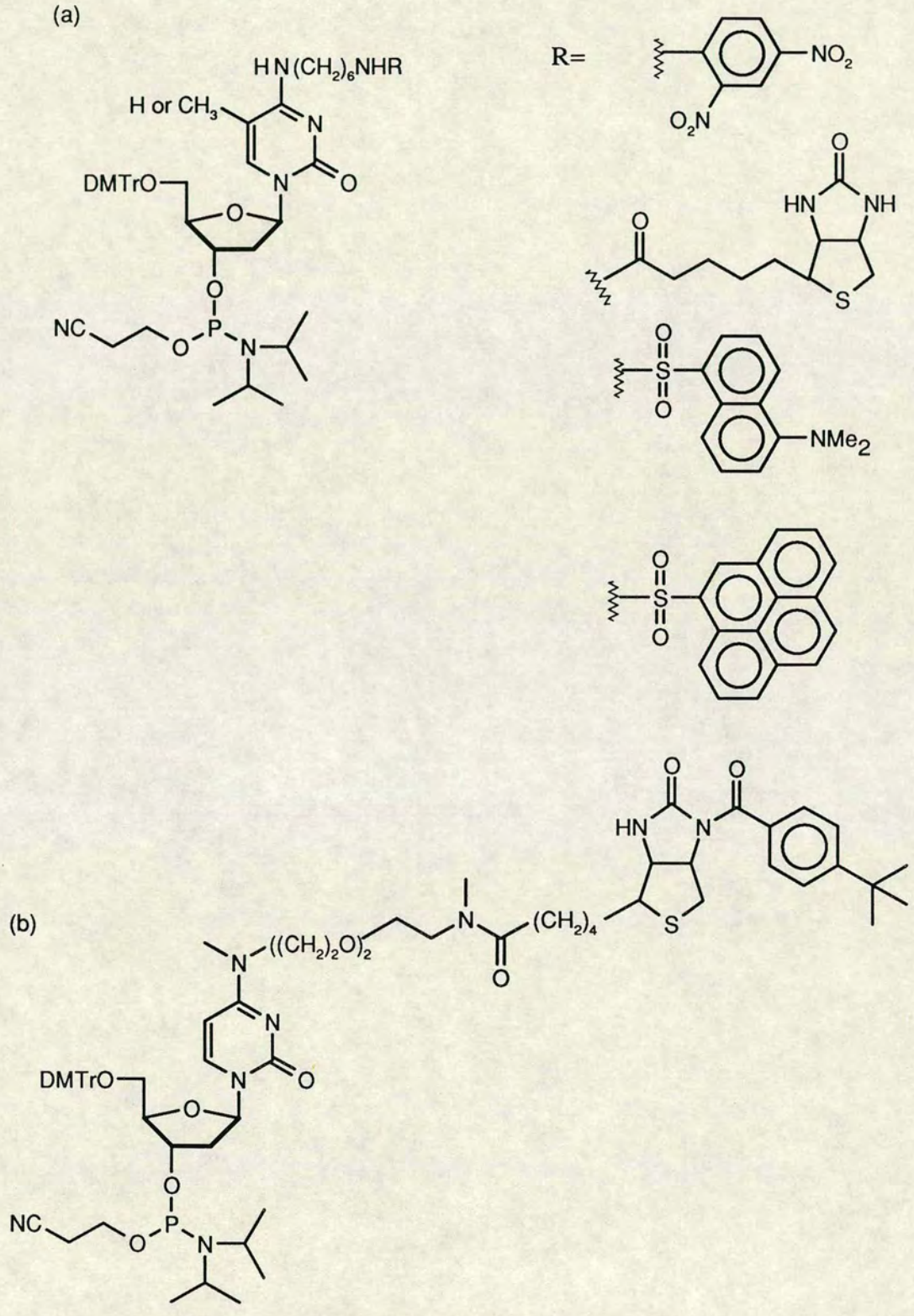


Figure 13: Nucleoside-based Labelling Phosphoramidites. (a) Roget et al. (ref. 91), (b) Pieles et al. (ref. 93).

The major drawbacks of both these methods, and of the modified nucleoside approach in general, are the expense of the starting materials and the large number of steps involved.

An alternative approach to the synthesis of labelling phosphoramidites has been used by Misiura *et al.* [27] Multiple biotin and phosphotyrosine groups were incorporated in oligonucleotides using a non-nucleoside phosphoramidite based on a 3-carbon glyceryl backbone (*Figure 14*). The anion of solketal was reacted with acrylonitrile, and the resulting 2-cyanoethyl solketal was reduced to 3-aminopropyl solketal using $NaBH_4/CoCl_2$. This amine was then used as the basic framework to synthesize both a biotin and a phosphotyrosine phosphoramidite. Reaction with Biotin-N-hydroxysuccinimide ester, followed by removal of the isopropylidene group with aqueous HCl, dimethoxytritylation of the primary hydroxyl, and phosphitylation of the secondary hydroxyl, gave the biotin phosphoramidite (*Figure 14a*). An analogous procedure was used to synthesize the phosphotyrosine phosphoramidite (*Figure 14b*), however the amine was reacted with the pentafluorophenyl ester of protected L-tyrosine, instead of the biotin active ester.

Standard streptavidin-AP conjugate detection showed disappointingly that oligonucleotides carrying multiple biotin labels gave the *same* detection sensitivity as those carrying a single biotin, even when the biotin residues were spaced apart using thyminidyl residues. This was attributed to the large size of streptavidin preventing binding of more than one streptavidin molecule to the group of biotin labels. Detection using an *anti*-biotin antibody followed by a secondary antibody-AP conjugate gave an enhanced signal for multiply biotinylated oligonucleotides. The reason for the enhanced signal when using *anti*-biotin antibody but not streptavidin is not clear.

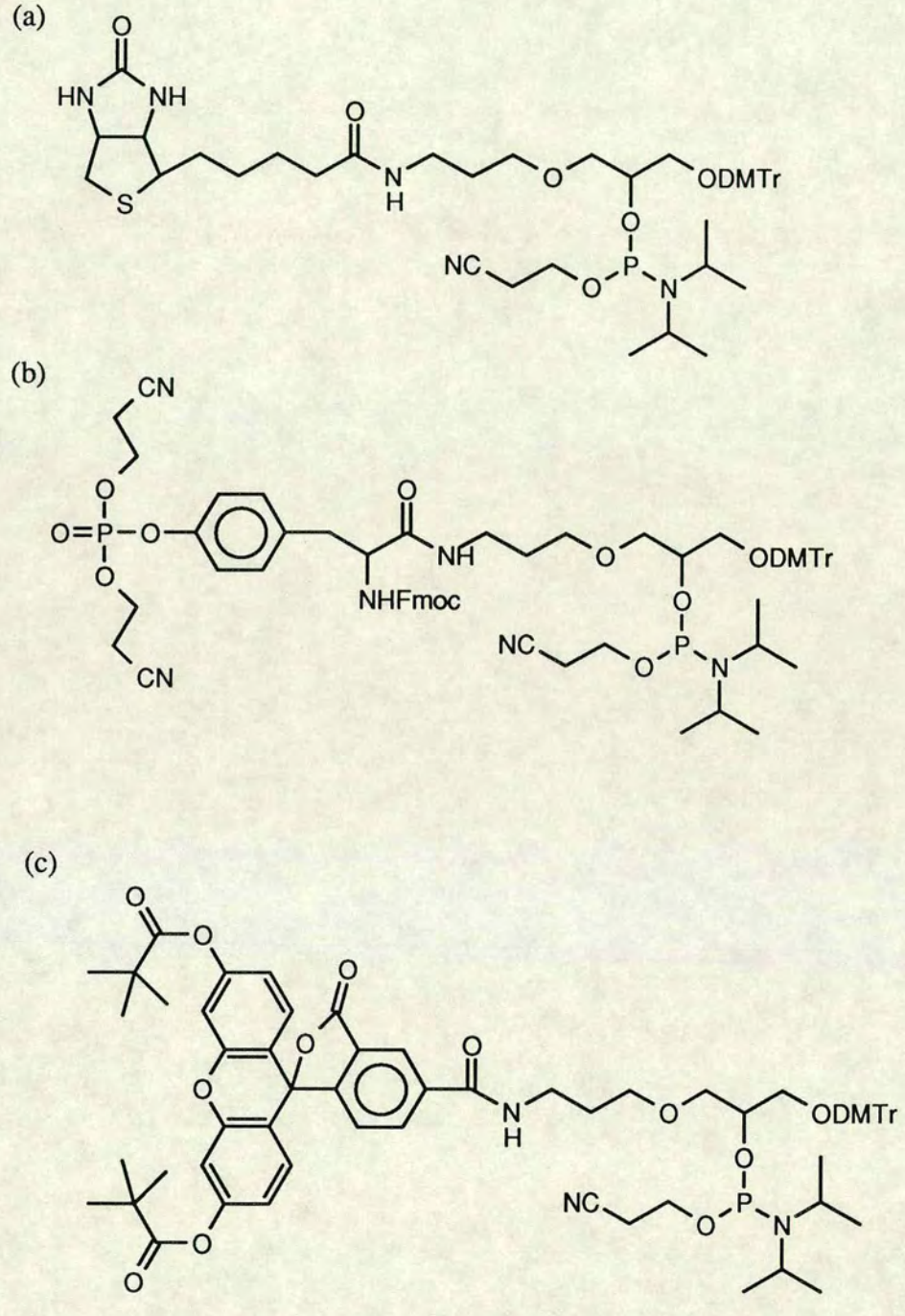


Figure 14: Multiple addition phosphoramidites based on the aminopropyl solketal backbone of Misiura et al. (ref. 27)
(a) Biotin phosphoramidite (ref. 27)
(b) Phosphotyrosine phosphoramidite (ref. 27)
(c) Fluorescein phosphoramidite (ref. 94).

Detection of phosphotyrosine labelled oligonucleotides was carried out using an anti-phosphotyrosine antibody followed by a secondary antibody-AP conjugate. The sensitivity of phophotyrosine detection was approximately 10 times lower than that of biotin, and the signal strength on increasing the number of labels did not rise as dramatically as for the biotin/*anti* -biotin antibody system.

Theisen *et al.* [94] have recently synthesized a multiple addition 5-carboxyfluorescein phosphoramidite (*Figure 14c*) using the same backbone as that used by Misiura *et al.* [27] This monomer is soluble in acetonitrile, and requires minimal changes to the oligonucleotide synthesis cycle for optimal coupling.

As with all phosphoramidites based on the 1,2-diol system of 3-aminopropyl solketal, the oligonucleotides must be synthesized with the 5-O-dimethoxytrityl group in place (Trityl-ON synthesis) to prevent label cleavage by attack of the free primary hydroxyl on the adjacent phosphodiester moiety under ammonia deprotection conditions.

4.0. Aims.

It is clear from the work described in this introduction that the future of non-radioactive labelling of oligonucleotides lies in the introduction of labels as phosphoramidites during automated solid-phase synthesis. The aim of the project described in this chapter was to develop inexpensive non-nucleoside phosphoramidite monomers for the routine synthesis of mono- and poly-labelled oligonucleotides.

B. RESULTS AND DISCUSSION.

5.0. Synthesis of 2,4-Dinitrophenyl-containing Phosphoramidites and their Use in Oligonucleotide Synthesis and Detection.

The basic requirements for a starting material for a phosphoramidite monomer suitable for poly-labelling of oligonucleotides with DNP groups are:

(i) It must contain at least one primary or secondary amino group for the attachment of the labelling group.

(ii) It must contain two hydroxyl functions, one for the attachment of the dimethoxytrityl group, the second for the phosphoramidite.

A suitable starting material for a DNP labelled monomer was found to be bis-(2-hydroxyethyl)-ethylene diamine (*Scheme 1*). This molecule has the following advantages:

(i) It contains two secondary amino functions, allowing the attachment of two DNP groups (or other labelling groups) to the molecule, thus two labels are attached to the oligonucleotide per addition during synthesis.

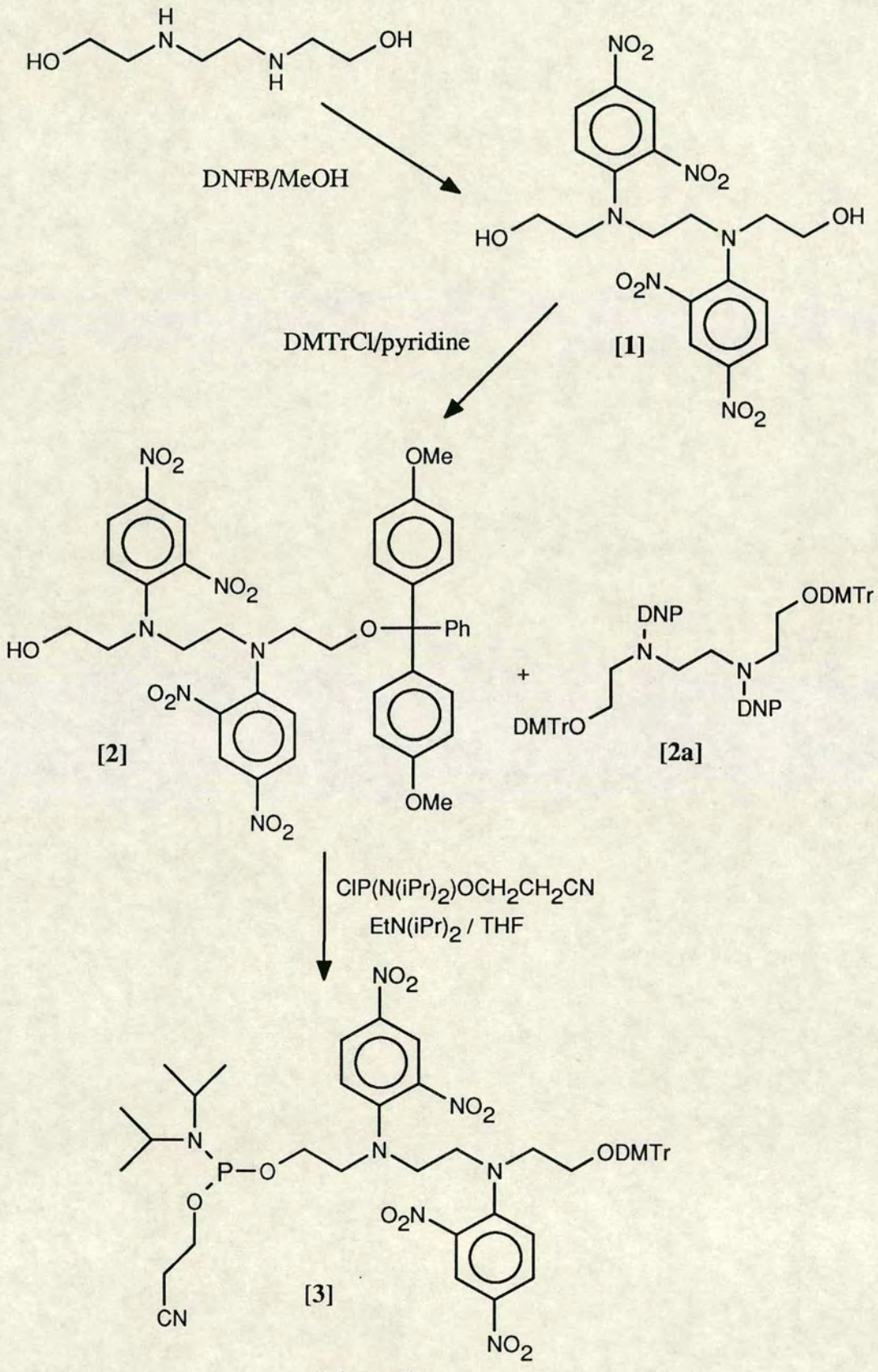
(ii) It is achiral.

(iii) It is inexpensive.

(iv) The hydroxyl groups are at opposite ends of the molecule, minimising any possibility of steric interaction between the bulky dimethoxytrityl group and the phosphoramidite group which may interfere with coupling.

(v) It is unlike any other molecule previously used to label oligonucleotides, and is thus unlikely to infringe existing patents.

The main disadvantage of this molecule is that it contains two primary hydroxyl functions, thus there is no selectivity in the tritylation reaction,


DNFB/MeOH
DMTrCl/pyridine
[1]
[2]
[2a]
[3]
ClP(N(iPr)2)OCH2CH2CN
EtN(iPr)2 / THF

Scheme 1

and the bis-(dimethoxytrityl) product **[2a]** is formed in addition to the required mono-(dimethoxytrityl) product **[2]**. It was decided to attempt the synthesis of a DNP labelled monomer based on this backbone.

5.1. *Chemical Synthesis.*

The synthetic route used is shown in *Scheme 1*. 2,4-dinitro-fluorobenzene (DNFB; **Caution:Highly Toxic**) was added to the starting material in the minimum volume of methanol in the presence of triethylamine to neutralise the HF generated in the reaction. The reaction was complete after 3.5 hours and the product crystallised from the reaction mixture. The product **[1]** was filtered-off and recrystallised from methanol. Larger single crystals of **[1]** were grown from diluted portions of the supersaturated hot liquor from the third recrystallisation. These crystals were used by Dr. Gordon Leonard to obtain an X-Ray crystal structure of **[1]** (*Figure 15*). The molecule was found to have an inversion centre, with the two DNP groups in an "anti" configuration with respect to each other. This increases the chances of antibodies binding to the DNP groups as there is no interaction between them. Any interaction between the DNP groups would be likely to impare antibody-hapten recognition and inhibit antibody binding.

The tritylation of **[1]** using 1 equivalent of dimethoxytrityl chloride in anhydrous pyridine resulted in a mixture of **[1]**, **[2]**, and the bis-(4,4'-dimethoxytrityl) adduct **[2a]**, even when a dilute solution of dimethoxytrityl chloride in pyridine was added dropwise over several minutes. It was decided to add 1.3 equivalents of dimethoxytrityl chloride to give a mixture of predominantly **[2]** and **[2a]**. These products are readily distinguished by tlc and are easily separated by flash chromatography. It may be possible to carry out a controlled deprotection

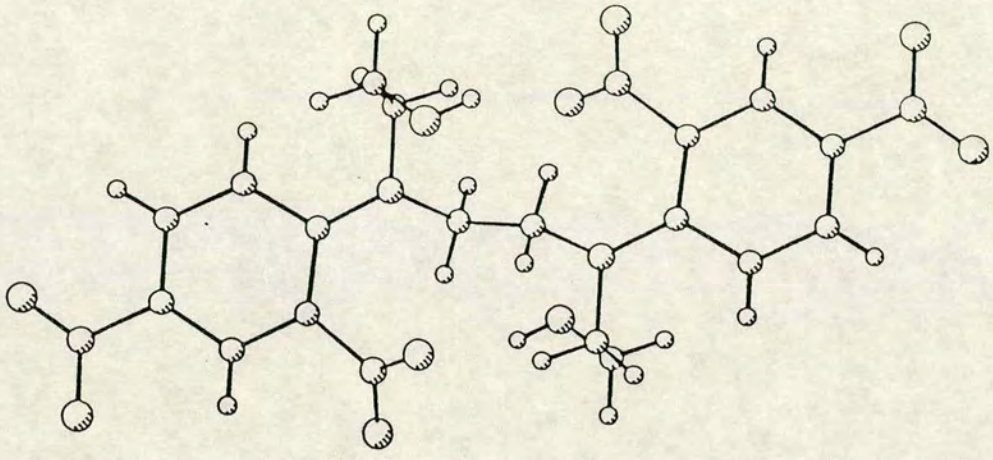
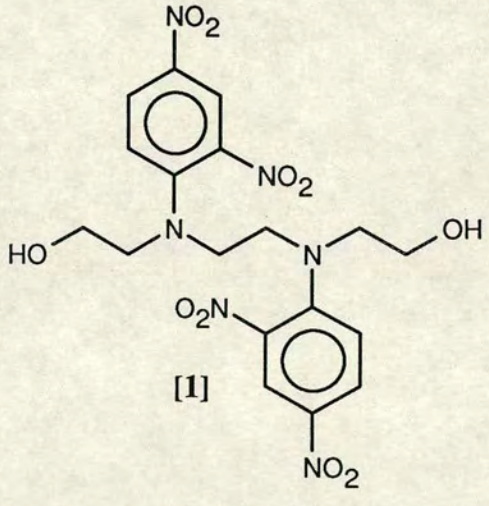


Figure 15: The X-ray crystal structure of [1].



[1]

of **[2a]** to generate **[2]** using a mild acid treatment, or alternatively **[1]** could be regenerated from **[2a]** by treatment with trichloroacetic acid, although this was not attempted.

The phosphitylation of **[2]** was carried out in anhydrous tetrahydrofuran using 2-cyanoethyl N,N-di-isopropylphosphoramido-chloridite. The reaction gave one product on tlc and after a rapid aqueous work-up this was purified by flash chromatography, using an eluent containing 1% triethylamine. The product **[3]** was obtained as a bright yellow foam. Oligonucleotide syntheses carried out using this foam gave coupling efficiencies which ranged from 88% to 95%. However, when this foam was dissolved in a minimum volume of dichloromethane and precipitated from hexane at -78°C, the resultant yellow powder gave coupling efficiencies of around 97%. This increase in coupling efficiency is probably due to the removal of residual triethylamine and other minor impurities which are left in solution in the hexane after precipitation.

5.2. *Oligonucleotide Synthesis using Standard DNA Synthesis Monomers.*

Compound **[3]** was found to be insoluble in anhydrous acetonitrile, the standard solvent for DNA synthesis monomers. Attempts to use anhydrous dimethylformamide as solvent gave low coupling efficiencies, and finally anhydrous dichloromethane was found to be the solvent of choice. To test for possible adverse effects of using dichloromethane as a solvent, a test sequence d(CpCpCpCpT) was synthesized using normal dC monomer dissolved in anhydrous dichloromethane. Average coupling efficiencies were found to be 98-99%, indicating that the use of dichloromethane as a solvent would not adversely affect coupling efficiency during DNA synthesis.

A series of short test sequences were synthesized to assess the coupling efficiency of **[3]** and to test its stability to standard synthesis and deprotection conditions. The synthesis cycle used was the standard ABI cycle used for 0.2 μmole scale DNA synthesis, with a normal 30 second coupling time. The DNP monomer **[3]** was used as a 0.15M solution in anhydrous dichloromethane and its coupling efficiency, determined by analysis of released dimethoxytrityl cations, was 97%. After cleavage from the solid support and ammonia deprotection the bright yellow oligonucleotides were analysed by HPLC.

5.3. *HPLC analysis of DNP-oligonucleotides.*

Oligonucleotides carrying DNP-groups were analysed and purified by reversed-phase HPLC. The HPLC characteristics of oligonucleotides containing the hydrophobic DNP labels were very different to those of unlabelled failure sequences. The DNP oligonucleotides had retention times between 5 and 15 minutes longer than unlabelled oligonucleotides, depending on the number of DNP groups incorporated. Oligonucleotides containing more than two DNP groups failed to elute from the HPLC column under normal oligonucleotide HPLC purification conditions (A gradient of 0-20% acetonitrile in 0.1M ammonium acetate). Thus it was necessary to increase the final acetonitrile concentration to 65%. The labelled oligonucleotides were bright yellow, making identification of the product relatively easy.

5.4. *Stability of the DNP label to synthesis and deprotection conditions.*

It is clear from the HPLC chromatograms shown in *Figure 16 (a), (b)* that the desired labelled oligonucleotides (labelled [A]) were present in low yield. This is inconsistent with the 97% coupling efficiency measured for **[3]** in these syntheses and it was clear that degradation of the DNP label had occurred during ammonia deprotection, resulting in the formation of a hydrophobic impurity (labelled [H]) and several peaks at shorter retention times. To confirm that the ammonia deprotection was responsible for the degradation of the label, the product (peak [A]) **[3]**pApGpCpT from *Figure 16(a)* was collected and subjected to a 17 hour treatment with concentrated ammonia solution at 55°C. The results of this experiment are shown in *Figure 17 (a)&(b).* Obviously severe degradation of the DNP label had occurred. The synthesis of a DNP_4 PCR primer 22mer was attempted, but the mixture of failure sequences and degradation products made HPLC purification impossible. It appears that the use of this DNP monomer **[3]** is incompatible with the harsh ammonia deprotection conditions required to remove the base protecting groups on standard DNA synthesis monomers.

To test the purity of a labelled oligonucleotide in ammonia at room temperature, a labelled test sequence **[3]**pTpTpTpT was synthesized. As T has no base protecting group the only post-synthetic step required is the cleavage of the oligonucleotide from the solid support with concentrated ammonia for 1.5 hours at room temperature. The HPLC Chromatogram of crude **[3]**pTpTpTpT is shown in *Figure 18.* The virtual absence of non-DNP containing failure sequences shows that the DNP monomer **[3]** couples extremely well and confirms that the only problem is its instability to prolonged ammonia treatment at 55°C.

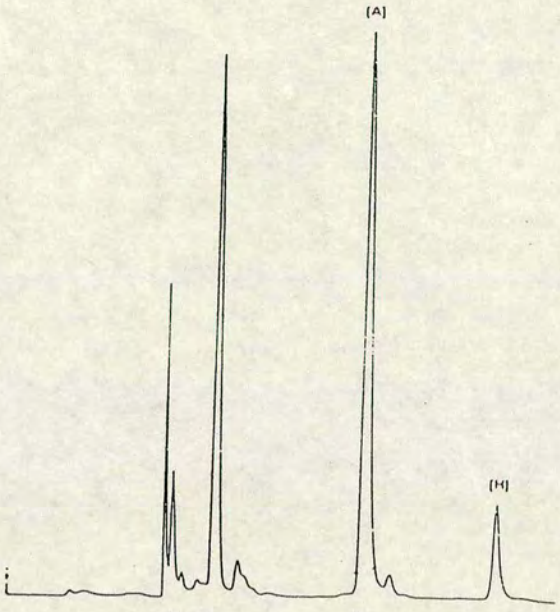


Figure 16(a): HPLC Chromatogram of crude [3]pApGpCpT *after 5h at 55°C in* NH_4OH. [A]=DNP oligonucleotide; [H]=hydrophobic degradation product.

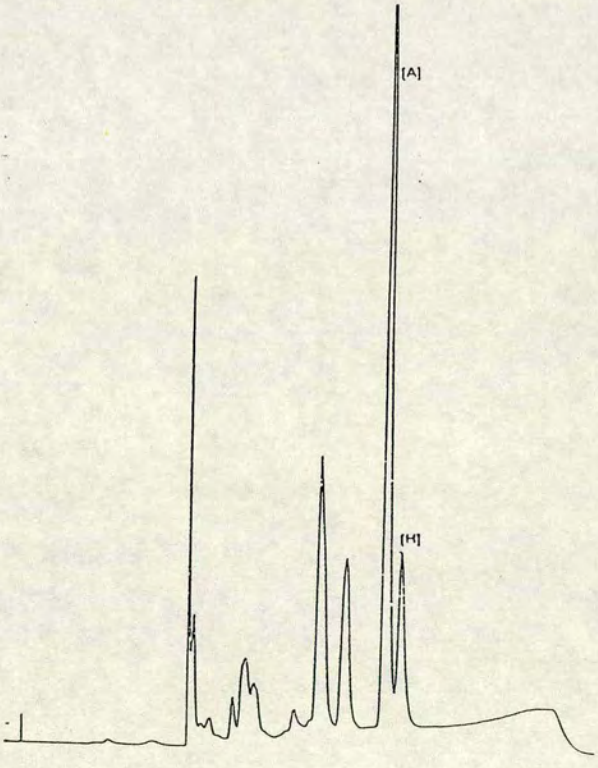


Figure 16(b): HPLC Chromatogram of crude [3]p[3]pApGpCpT *after 5h at 55°C in* NH_4OH. [A]=DNP oligonucleotide; [H]=hydrophobic degradation product.

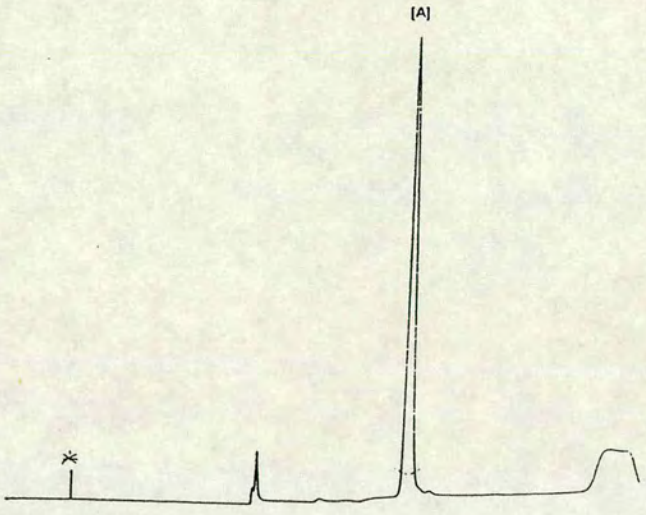


Figure 17(a): HPLC Chromatogram of purified [3]pApGpCpT *prior to ammonia treatment.* [A]=DNP oligonucleotide.

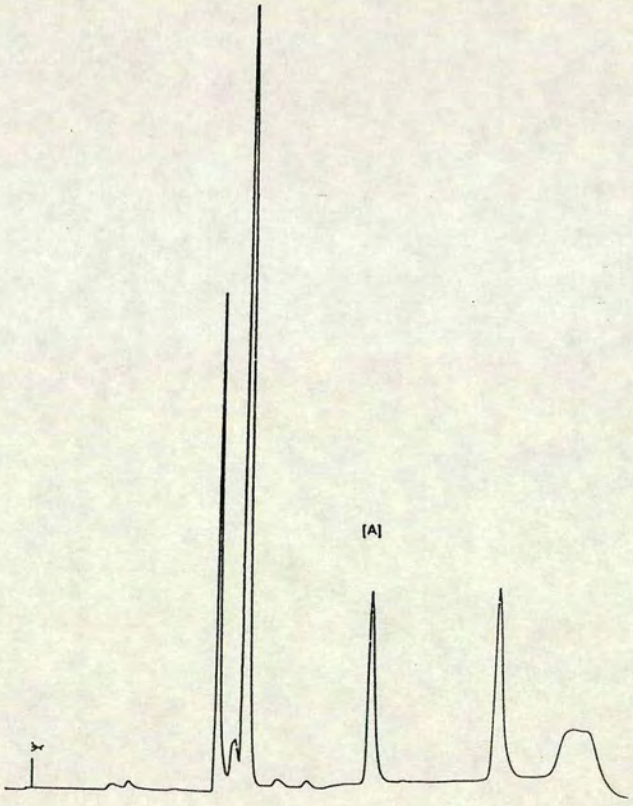


Figure 17(b): HPLC Chromatogram of purified [3]pApGpCpT *after treatment with* NH_4OH *at 55°C for 17 hours.* [A]=DNP oligonucleotide.

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5.5 Oligonucleotide Synthesis using 'PAC' DNA Synthesis Monomers.

The encouraging result of the synthesis of **[3]**pTpTpTpT showed that the DNP label was stable to ammonia treatment at 20°C. It was decided to attempt to synthesize DNP labelled oligonucleotides using **[3]** in conjunction with DNA monomers with base protecting groups which are more labile to concentrated ammonia. These commercially available "PAC Amidites" [92] are essentially the same as normal DNA monomers, except that the base protecting group for dA and dG is the phenoxyacetyl group and for dC the isobutyryl group. The conditions required for the removal of these protecting groups are either 5 hours in concentrated ammonia at 20°C; or 1-2 hour in concentrated ammonia at 55°C. It was hoped that the DNP labels would be stable under these conditions. Unfortunately the manufacturer's instructions for the use of the PAC monomers were erroneous, and the dG PAC monomer was only partially soluble in the anhydrous acetonitrile/anhydrous dimethylformamide mixture recommended. However, the dG PAC monomer was found to be highly soluble in anhydrous dichloromethane, and this solvent was used in all subsequent syntheses.

HPLC analysis of DNP-oligonucleotides synthesized using PAC amidites indicated that the DNP labels were largely stable to the milder deprotection conditions used. The HPLC chromatogram of a crude oligonucleotide labelled with 6 DNP groups after 1 hour in concentrated ammonia at 55°C is shown in *Figure 19*. The overall yield for this synthesis was 83% and the chromatogram shows very little evidence of degradation of the DNP label. The sample was passed through a size exclusion Sephadex G25 (NAP) column which retains any small molecules (*eg.* degradation products) and allows the passage of larger molecules (*eg.* oligonucleotides). A very faint yellow band was left in

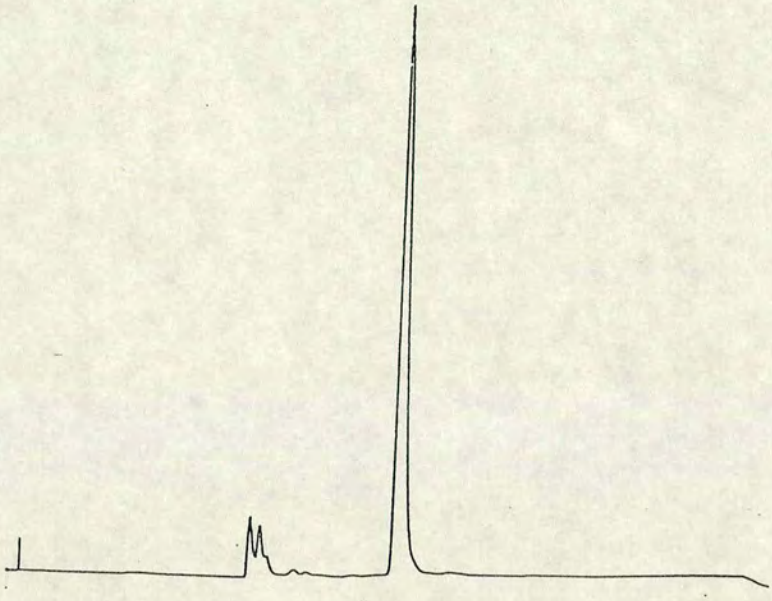


Figure 18: HPLC Chromatogram of crude [3]pTpTpTpT.

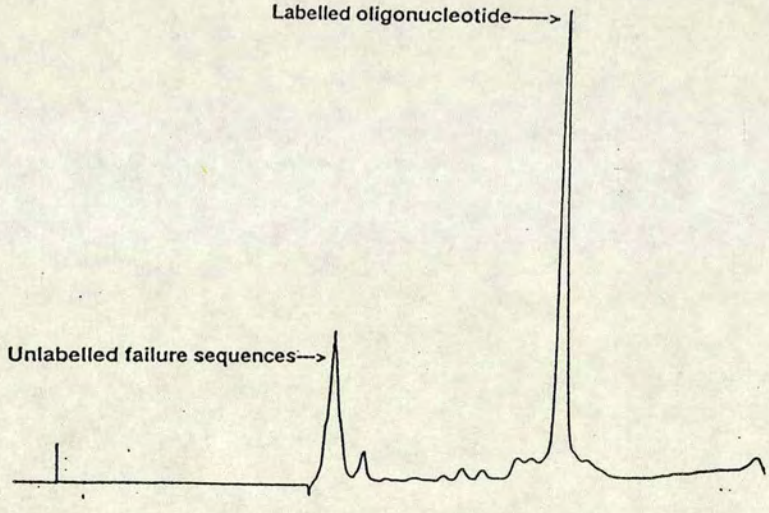


Figure 19: HPLC Chromatogram of a crude DNP_6 *18mer oligonucleotide.*

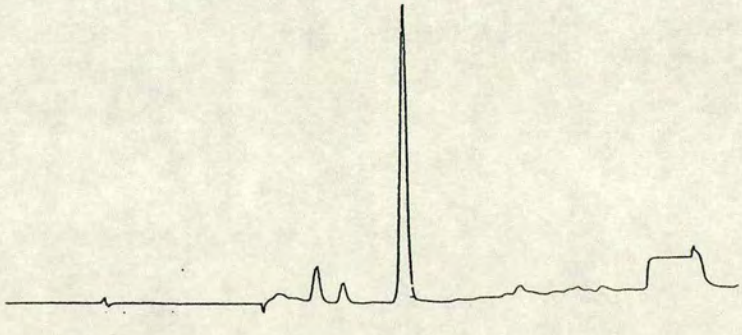


Figure 20: HPLC Chromatogram of degradation product of DNP label.

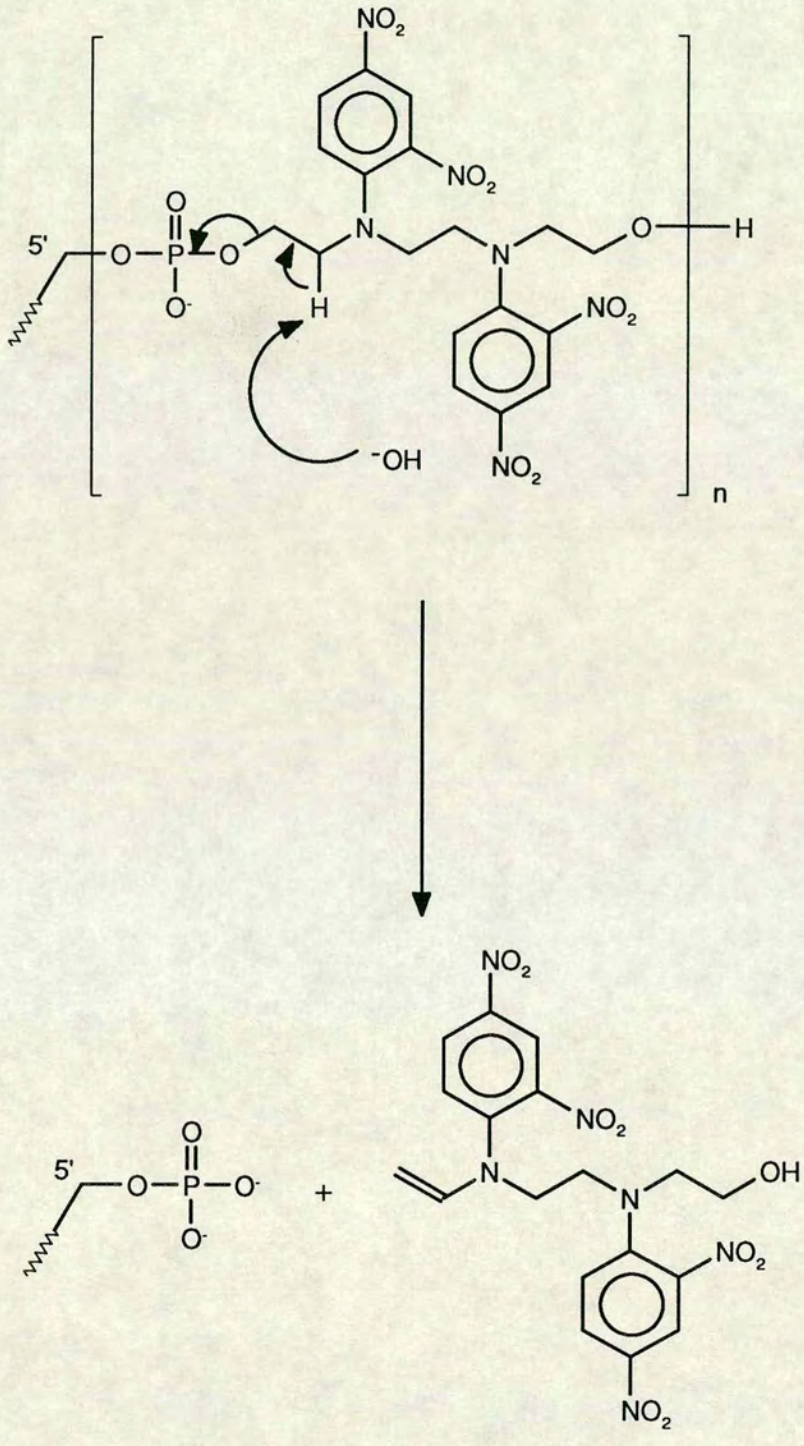


Figure 21: Possible Mechanism of DNP Label Degradation

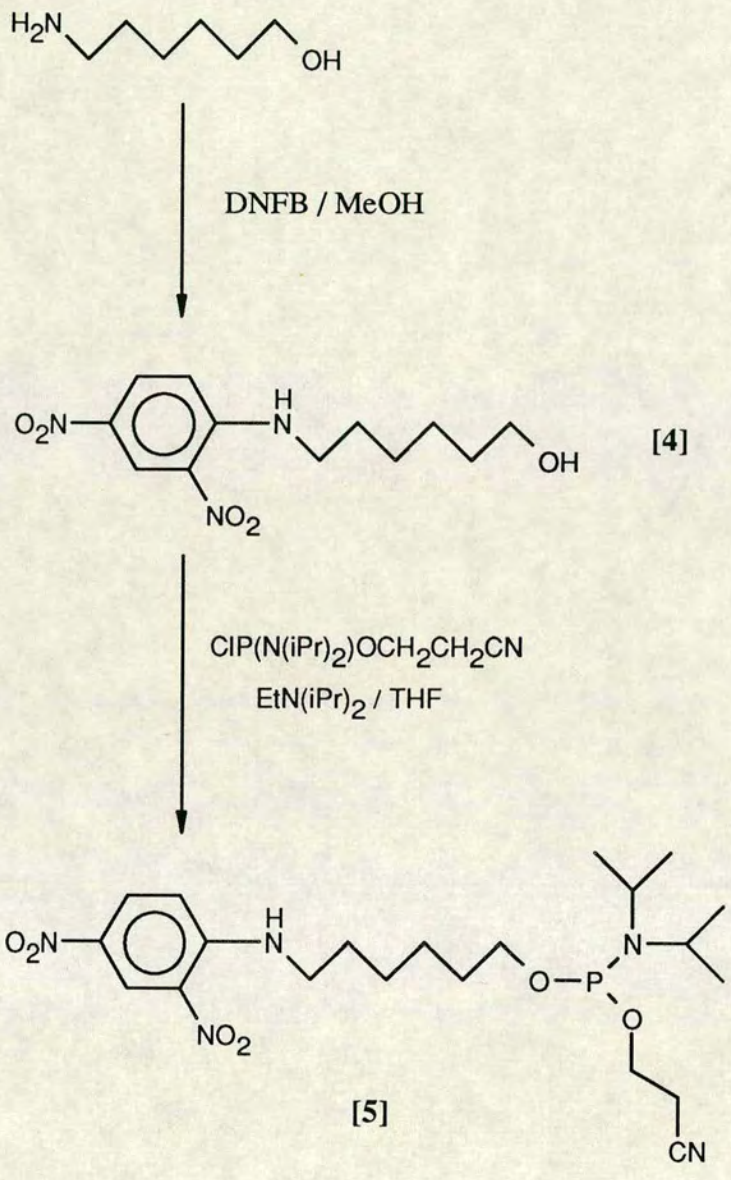
the column after elution of the oligonucleotide-containing fraction and this was eluted and collected for HPLC analysis. The HPLC chromatogram of this impurity collected from the NAP column is shown in *Figure 20* . The degradation of DNP substituted primary amines in aqueous ammonia has been reported by Roget *et al.* who identified dinitrophenol as the product of this decomposition[91]. Peak enhancement injections of solutions of 2,4-dinitroaniline and 2,4-dinitrophenol mixed with the impurity in solution showed that the major impurity was neither of these compounds. The labelled oligonucleotides synthesized using **[3]** contain DNP derivatised secondary amino functions and a possible mechanism for label degradation is shown in *Figure 21* . To date no evidence for this mechanism has been collected. In order to obtain an estimate for the rate of degradation in aqueous ammonia at 20°C, a PCR primer (24mer, DW1, Appendix 1) monolabelled with **[3]** was synthesized and purified by HPLC. A sample of this purified oligonucleotide was treated with concentrated ammonia solution for 5 hours at 22°C, removing aliquots every hour for HPLC analysis. The HPLC Chromatograms show no evidence of degradation of the label under these conditions.

5.6. *Synthesis of a Single Addition DNP phosphoramidite.*

The manner in which the DNP hapten is presented for recognition by the antibody may affect the kinetics and thermodynamics of the antibody-hapten interaction. It was therefore decided to synthesize a DNP phosphoramidite in which the DNP group was separated from the 5'-end of the oligonucleotide by a spacer arm. Such a phosphoramidite had already been synthesized in this research group by Pritchard.[88] The synthesis was carried-out largely as described previously(*Scheme 2*). 2,4-

Dinitrofluorobenzene was reacted with 6-amino-hexan-1-ol in the presence of triethylamine in methanol. The 6-(2,4-dinitrophenylamino)-hexan-1-ol **[4]** was crystallised from diethyl ether /methanol. It tended to form an insoluble oil on heating with the crystallisation solvents, but eventually analytically pure crystals were obtained. Grzybowski (*unpublished results*) found that recrystallisation from hexane\ethyl acetate gave more satisfactory results. Phosphitylation of **[4]** proceeded smoothly in tetrahydrofuran to give the single addition DNP phosphoramidite **[5]**. As reported by Pritchard, the phosphoramidite was soluble in acetonitrile, and when used as a 0.1M solution it was found to couple with an efficiency of >95% (as estimated by HPLC), under standard DNA synthesis conditions. The DNP group on this phosphoramidite was found to be *stable* to ammonia deprotection conditions. Oligonucleotides labelled with **[5]** could be purified under standard HPLC conditions[81] and eluted approximately 2 minutes after unlabelled failure sequences.

A large number of Polymerase Chain Reaction (PCR) primers and *in situ* hybridisation probes labelled with 1, 2, 6 and 10 DNP groups were synthesized using **[3]** and **[5]** and purified for the use of collaborators in a variety of applications. We await the results of these studies. An *anti*-DNP antibody was supplied by Dr. Rick Randall of the Biochemistry Dept. of St. Andrews University. This antibody was raised in mice, by immunising with dead *E.Coli* bacteria which had been reacted with 2,4-dinitrofluorobenzene. It was decided to attempt some antibody detection experiments during a two week visit to the laboratories of Dr. Randall.


H2N
OH
DNFB / MeOH
O2N
H
N
OH
NO2
[4]
ClP(N(iPr)2)OCH2CH2CN
EtN(iPr)2 / THF
O2N
H
N
N
O
P
O
NO2
CN
[5]

Scheme 2

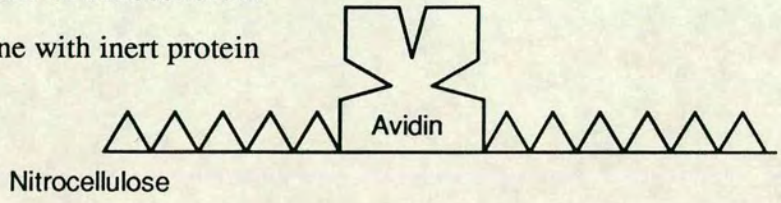
5.7. Polymerase Chain Reaction using DNP-labelled Primers.

Amplification of specific sequences in genomic DNA using Polymerase Chain Reaction (PCR)[95,96] has become an important tool in many areas of biological and medical research. PCR is particularly useful for the detection of single-copy mutations which cause a number of inherited diseases, such as Cystic Fibrosis and Sickle Cell Anaemia.[1] Without PCR amplification the detection of such mutations would be very difficult or impossible. The use of 'Capture and Detect' experiments on PCR products opens the way for large-scale diagnostic testing using PCR technology. (*Figure 22 (a) and (b)*) The 'Capture and Detect' format requires the synthesis of hapten or biotin labelled PCR primers. It is thus essential that any new hapten label must not inhibit PCR when it is incorporated into a PCR primer. It was therefore essential to test PCR primers labelled with single and multiple DNP groups.

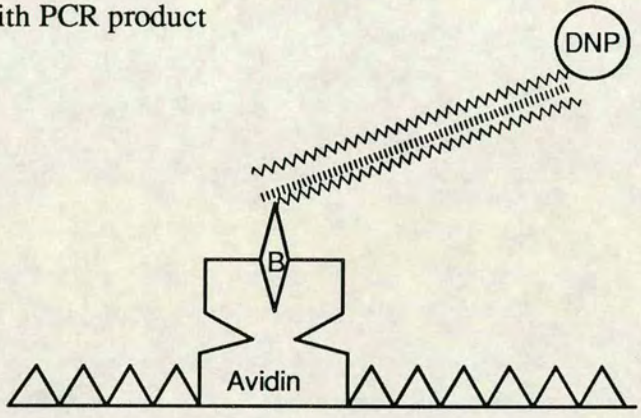
PCR primers labelled with 1, 2, 6, and 10 DNP groups were synthesized using **[3]** and **[5]** (*Figure 23 a* primer A) . A biotinylated (Bio) primer (*Figure 23 a* primer B) was synthesized by reaction of Biotin-N-hydroxysuccinimide ester with the aminomodified primer (Aminolink 2). In addition a PCR primer was synthesized which incorporated alternating hexaethyleneglycol-**[5]** units. The hexaethyleneglycol (heg) units were introduced using a heg phosphoramidite[97] (*Figure 23 b*). (A gift from Susanne Ebel). Using the Alchemy II molecular graphics software (Tripos Assoc.; IBM PC) it was found that when fully extended the hydrophilic heg units introduce a 23.3Å spacer between the DNP labels. The primers were for the PCR amplification of a section of the HN gene of paramyxovirus. This PCR system had been optimised using radiolabelled primers in the laboratories of Dr. Randall, and was used as a convenient test of the applicability of

(1) Immobilise avidin on nitrocellulose

(2) Block membrane with inert protein



(3) Incubate with PCR product



(4) Incubate with anti-DNP antibody, then secondary antibody-enzyme conjugate

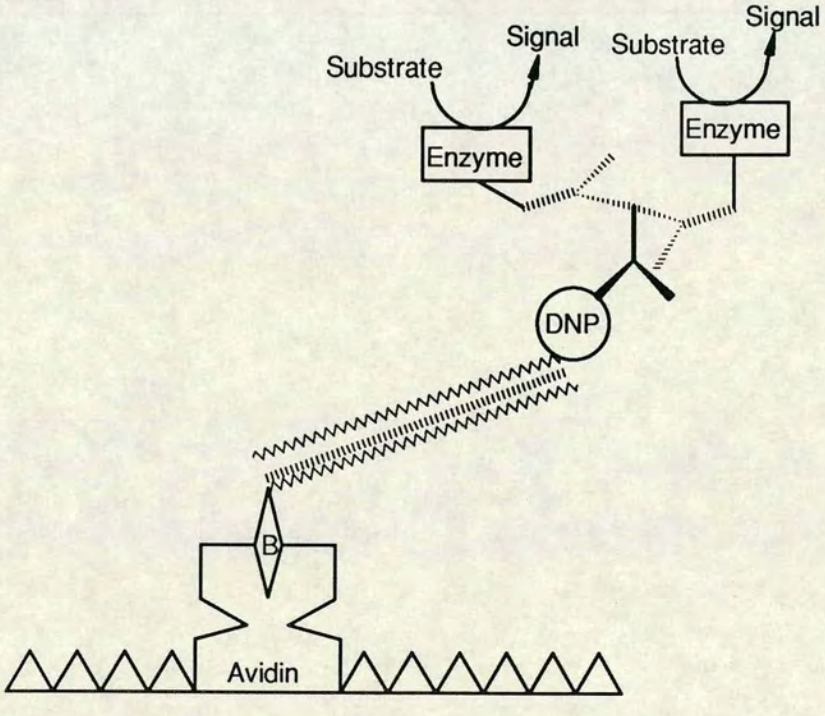


Figure 22(a): Schematic diagram showing the Capture and Detection of a double-stranded PCR product synthesized using a biotinylated and a DNP-labelled primer. (not to scale)

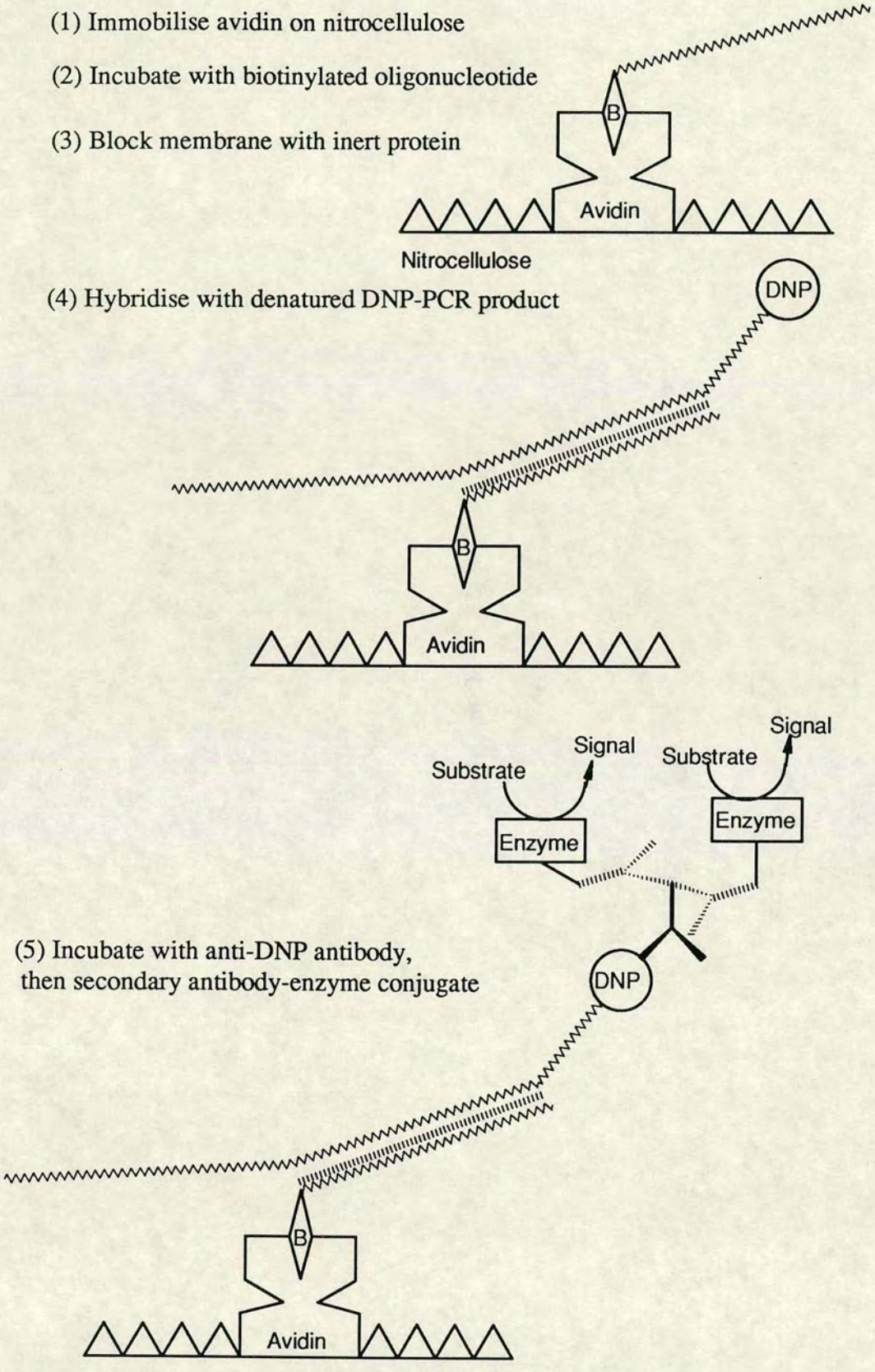
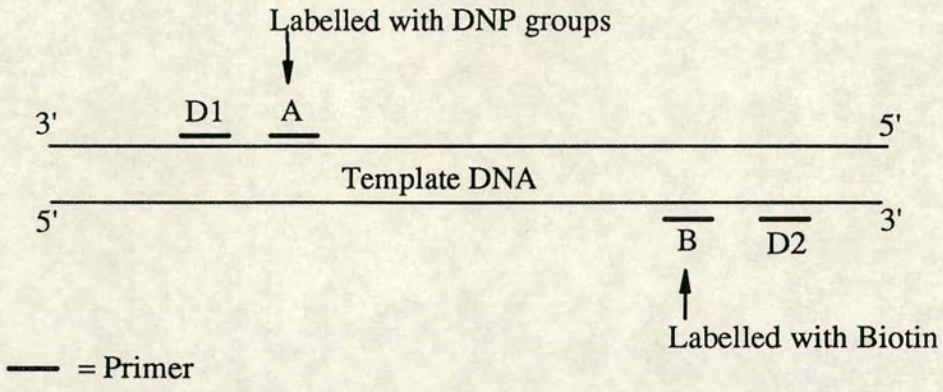
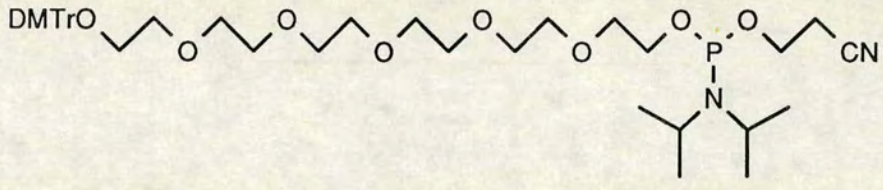


Figure 22(b): Schematic diagram showing the Reverse Dot Blot format for Capture and Detection of PCR Products.



(a) Relative primer positions in the PCR experiments.



(b) Hexaethylene glycol phosphoramidite, synthesized by Susanne Ebel

Figure 23

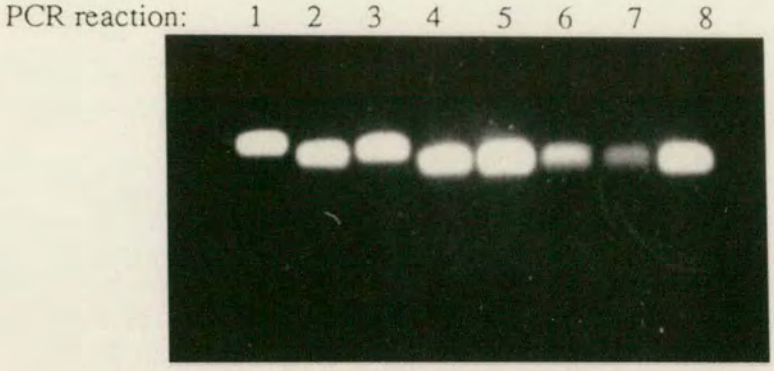
DNP primers to PCR. The PCR reactions were carried out under sterile conditions using standard procedures.[98] Primer concentrations were determined spectrophotometrically at 260nm using the extinction coefficients for DNA bases reported in the literature.[99] The extinction coefficients at 260nm (in methanol) of **[1]** and **[4]** ($9234M^{-1}cm^{-1}$ and $8872M^{-1}cm^{-1}$ respectively) were used to determine the UV absorbance of the DNP groups. A single strand hypochromicity factor of 0.9 was assumed. The following combination of primers was used:

PCR Reaction	Primer 1	Primer 2
1	D1	D2
2	A	D2
3	D1	BioB
4	DNP_1A	BioB
5	DNP_2A	BioB
6	DNP_6A	BioB
7	$DNP_{10}A$	BioB
8	$((DNP_2)heg)_2A$	BioB

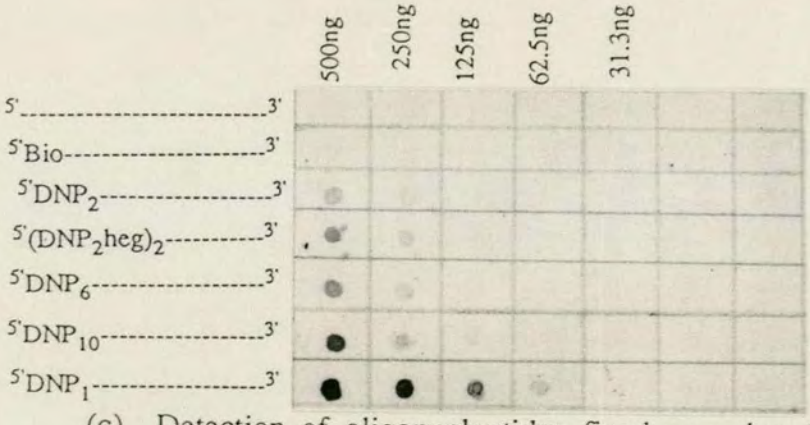
Where A, B, D1 and D2 are the primers shown in *Figure 23 a.* The sequences of these primers are given in Appendix 1.

The crude PCR reaction mixtures were analysed by Agarose gel electrophoresis, using ethidium bromide staining. The results of the PCR reactions are shown in *Figure 24 (a).* It is clear that the PCR reaction has been inhibited by the presence of 6 and 10 DNP groups at the 5'-end of the oligonucleotide. This experiment was repeated several times to confirm the finding. Doubling the concentration of the DNP primers in the reaction had no effect on the result. There are two possible reasons for the inhibition of PCR:

(a) Agarose gel of PCR reactions using Primers labelled with biotin, [3] or [5].



(b) Oligonucleotides fixed onto nylon and detected using *anti*-DNP antibody/secondary antibody-HRP conjugate.



(c) Detection of oligonucleotides fixed on nylon (using Avidin-HRP conjugate).

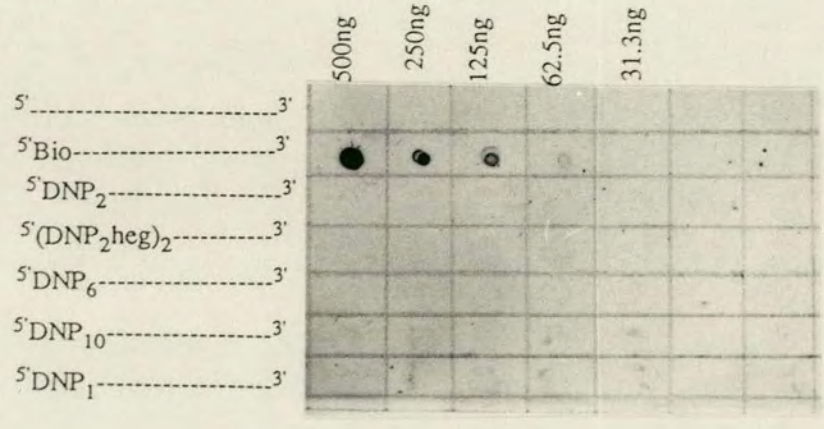


Figure 24

(i) The multiple DNP groups may inhibit the hybridization of the primer to the template strand.
(ii) The steric bulk of the multiple DNP labels may prevent the *Taq* Polymerase from reaching the 5'-end of the template strand, giving rise to truncated PCR products. This would eventually lead to termination of amplification as the primers would be unable to hybridize to the truncated PCR product template in the next PCR cycle. PCR was not inhibited by the presence of 4 DNP groups spaced using heg.

The failure of PCR using multiple DNP labelled primers is a serious problem, and rules out the use of **[3]** as a practical option for the introduction of multiple DNP groups into oligonucleotides for use as PCR primers. The use of heg units to space the DNP groups away from the 5'-end of the oligonucleotide may solve this problem, however the convenience of using a single DNP phosphoramidite for multiple labelling would be removed.

The problem of inhibition of PCR amplification due to the presence of multiple labelling groups at the 5'-end of oligonucleotides has not been reported previously. This is probably due to the fact that the use of multiply labelled oligonucleotides as PCR primers has seldom been reported. It was hoped that the problems encountered using multiple additions of **[3]** were due to the large size and relatively compact nature of **[3]** and that smaller DNP labelling groups incorporating longer spacer arms would eliminate the problem.

5.8. *Detection of DNP labelled oligonucleotides.*

Although the use of labelled oligonucleotides in PCR applications is undoubtedly important there are many other applications, such as the detection of viral diseases in clinical samples by *in situ* hybridisation of labelled probes, where the use of oligonucleotides labelled with **[3]** could be of potential use. In experiments to determine the suitability of the oligonucleotides labelled with **[3]** as probes we attempted to detect the DNP labelled PCR primers immobilised on nylon or nitrocellulose membranes using mouse monoclonal *anti*-DNP antibodies. Oligonucleotides were fixed onto nylon membranes by UV irradiation for 5 minutes, and onto nitrocellulose by baking in a vacuum oven at 80°C for 2 hours. The experiments were designed to determine the relative sensitivity of detection of different numbers of DNP labels and a Biotin label, rather than to obtain maximum absolute sensitivity.

5.8.1. *Selection of Anti-DNP antibody.*

Each labelled oligonucleotide was spotted and fixed onto five nylon membranes at a loading of 100ng. The filters were blocked with ovalbumin and each membrane was incubated with a 1:200 dilution of four ascitic fluids containing different *anti*-DNP antibodies. After washing and incubation with goat-*anti*-mouse IgG antibody-HRP conjugate, the DNP primers were detected using *o*-dianisidine. The strongest signal was obtained from the antibody with the designation K3. The ascitic fluid containing the K3 antibody was used in all subsequent experiments.

5.8.2. *Comparison of Sensitivity of detection.*

Five-fold dilutions of the labelled oligonucleotides were spotted and fixed in duplicate onto nylon membranes starting at 500ng loading. After blocking with ovalbumin, one membrane was incubated with streptavidin-HRP conjugate, and the other with K3 antibody followed by goat-*anti*-mouse IgG antibody-HRP conjugate. Both membranes were then developed with *o* -dianisidine. *Figure 24(b)* shows that all the multiple DNP labelled oligonucleotides (labelled with **[3]**) gave *poorer* sensitivity than the singly labelled oligonucleotide (labelled with **[5]**), and that the single DNP labelled oligonucleotide gives comparable sensitivity to the biotin labelled oligonucleotide under the experimental conditions used. The probable reason for the drop in sensitivity for the multiple DNP oligonucleotides compared to the single DNP oligonucleotide is that the antibody does not recognise an unspaced DNP group attached to a secondary amine. The oligonucleotide with 10 DNP groups gives only slightly higher sensitivity than those with 6 or 4(spaced) DNP groups, but gives a much stronger signal than the oligonucleotides with 2 DNP groups. This indicates that multiple DNP labelling does give an inprovement in sensitivity, however the manner in which the DNP groups are presented for antibody binding is of crucial importance.

The results of the experiments described above show that DNP labelling of oligonucleotides using **[3]** is fundamentally impractical.

5.9. ***Conclusions about*** **[3]** ***and*** **[5].**

The following conclusions may be drawn from the work described above:

(a) The DNP monomer **[3]** is readily synthesized in reasonable yield from inexpensive starting materials in only three steps. This is a considerable

improvement over the only previous multiple addition DNP phosphoramidite[91] which required seven-step syntheses using relatively expensive starting materials.

(b) The major disadvantage of the synthetic route to **[3]** is the lack of selectivity during the tritylation, resulting in a low yield for this reaction.

(c) The coupling efficiency of **[3]** of 97% is reasonably good and could probably be improved by further purification.

(d) Two DNP labels are introduced per addition of **[3].** This cuts down on the amount of DNP monomer and machine time required for poly-labelling as the number of couplings is reduced.

(e) The degradation of the label during base deprotection has been considerably reduced by the use of DNA monomers with base protecting groups which are more base-labile, however these DNA monomers are prohibitively expensive for routine use. The mechanism for the degradation has not been elucidated.

(f) The insolubility of **[3]** in acetonitrile is a major drawback to the routine use of this phosphoramidite.

(g) Oligonucleotides which have been poly-labelled with DNP groups are very water-soluble in spite of having a large number of hydrophobic residues.

(h) The purification of DNP labelled oligonucleotides is very easy, as the HPLC characteristics of oligonucleotides containing the hydrophobic DNP labels are very different to unlabelled failure sequences. The labelled oligonucleotides are also bright yellow, making identification of the product relatively easy.

(i) The failure of PCR using primers labelled with **[3]** makes the use of **[3]** for PCR impractical. This effectively destroys the practical value of **[3]** for the many applications involving PCR.

(j) The failure of the anti-DNP antibody to recognise the DNP groups introduced by **[3]** rules out the use of **[3]** for *any* practical applications. This could not have been foreseen, and could be overcome by raising specific antibodies to proteins conjugated to **[3]**. However this is a major undertaking and is outside the scope of the present study.

6.0. Synthesis and Use of a Multiple-Addition DNP Phosphoramidite based on 1,2,6-Trihydroxyhexane.

The results described above help to define more precisely the requirements for a suitable backbone for a multiple DNP phosphoramidite. These requirements are:

(i) It must contain at least one primary but not secondary amino group for the attachment of the labelling moiety. A DNP group attached to the primary amino function of **[5]** was readily recognised by the antibody.

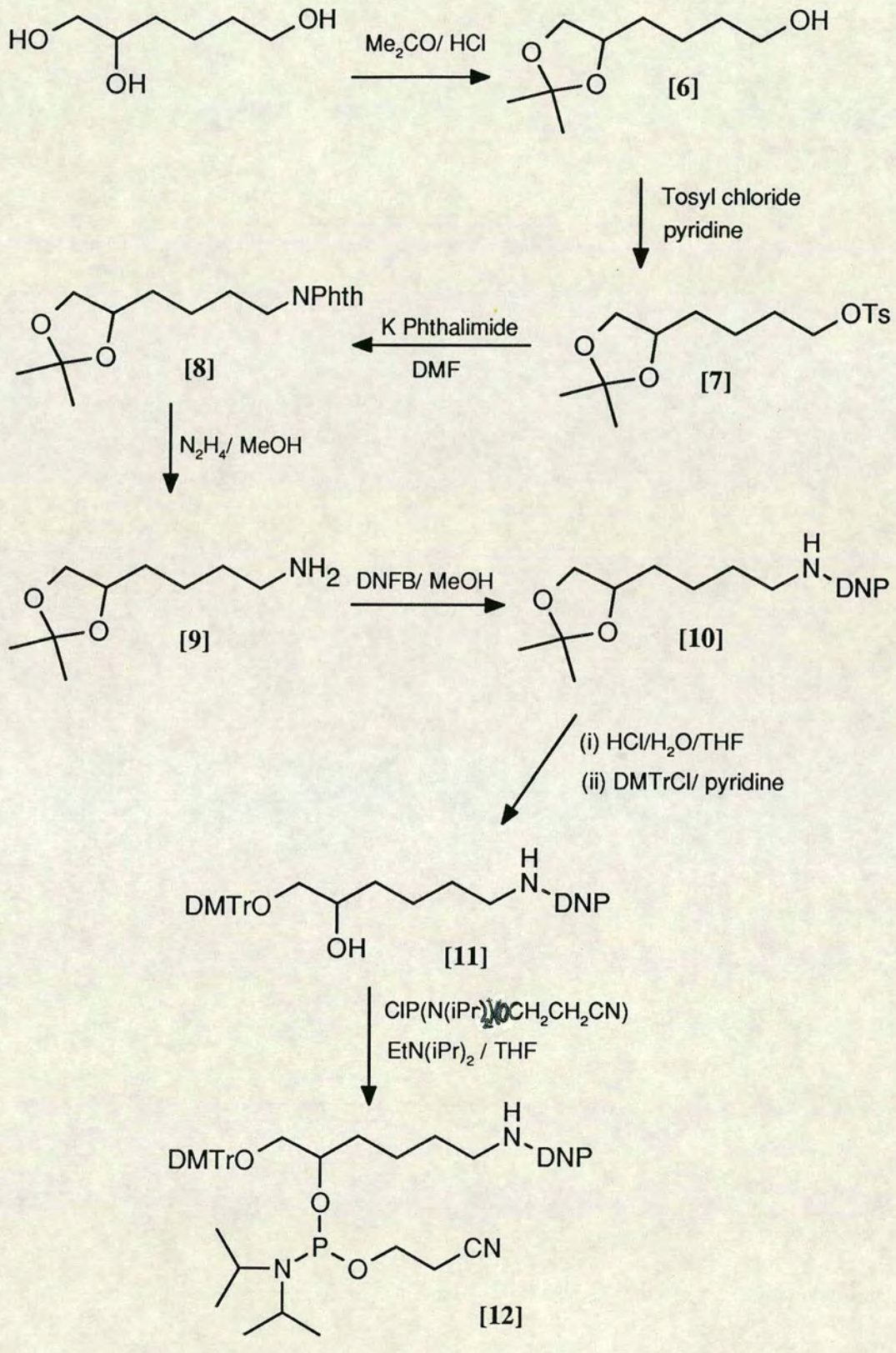
(ii) There must be a spacer arm between the DNP group and the rest of the molecule to facilitate antibody binding and minimise interactions between the DNP groups and the oligonucleotide which may inhibit PCR.

(iii) It must contain two hydroxyl functions, preferably one primary hydroxyl for the attachment of the dimethoxytrityl group and a secondary hydroxyl function for the phosphoramidite.

The use of the 3-aminopropyl solketal backbone of Misiura *et al.* [27] was considered (and is suggested for use with the DNP group in that reference) and John Grzybowski of this research group utilised this backbone as the basis for two DNP phosphoramidites. Another inexpensive starting material suitable as a backbone is 1,2,6-trihydroxyhexane, and it was decided to synthesize a DNP phosphoramidite based on this molecule.

6.1. *Chemical Synthesis.*

The synthetic route to the DNP phosphoramidite based on 1,2,6-trihydroxyhexane is shown in *Scheme 3*. Thus 1,2,6-trihydroxyhexane was converted to its acetonide derivative **[6]** (following a procedure based on that of Fischer and Phthaler[100]) by reaction with anhydrous acetone containing anhydrous sodium sulphate and a trace of concentrated aqueous HCl. The reaction was quenched by the addition of lead carbonate and the product was purified by kugelrohr distillation. It was found to be beneficial to add a trace of potassium carbonate to the distillation flask to ensure basic conditions to prevent decomposition of the product. The acetonide **[6]** was then tosylated (using a procedure based on that of Mori[101]). The tosylate synthesized by Mori was not characterised or purified, but the tosyl acetonide **[7]** was purified by flash chromatography and fully characterised. The tosyl group was displaced by potassium phthalimide in DMF, to give the phthalimide **[8]**. Removal of the phthaloyl group group using hydrazine hydrate (**Caution:Highly Toxic**) in methanol, gave after distillation the amine **[9]** as a clear liquid. This amine was then reacted with 2,4-dinitrofluorobenzene in methanol in the presence of triethylamine to give, after flash chromatography, the DNP-acetonide **[10]**. Previous work on related compounds by John Grzybowski had shown that deprotection of an acetonide and isolation of the resulting DNP-diol by flash chromatography resulted in extremely low yields due to the irreversible retention of the highly polar DNP-diol on the silica gel. Therefore the DNP acetonide **[10]** was converted to the dimethoxytrityl compound **[11]** in a 'one-pot' reaction. The acetonide protection was removed by treatment with aqueous HCl in THF. After exhaustive removal of traces of HCl and water by azeotropic evaporation with ethanol, toluene and anhydrous pyridine, the diol was reacted with


HO
OH
OH
Me_2CO/ HCl
[6]
Tosyl chloride
pyridine
NPhth
[8]
K Phthalimide
DMF
OTs
[7]
N_2H_4/ MeOH
NH_2
[9]
DNFB/ MeOH
H
N
DNP
[10]
(i) HCl/H_2O/THF
(ii) DMTrCl/ pyridine
DMTrO
OH
[11]
ClP(N(iPr)$_2$)OCH$_2$CH$_2$CN)
EtN(iPr)$_2$ / THF
P
CN
[12]

Scheme 3

4,4'-dimethoxytrityl chloride in pyridine to give the phosphoramidite precursor **[11]**. Phosphitylation of **[11]** in anhydrous THF, using 2-cyanoethyl N,N-diisopropylphosphoramidochloridite in the presence of N,N-diisopropylethylamine, proceeded smoothly to give the multiple-addition DNP phosphoramidite **[12]** as an orange oil.

6.2. *Oligonucleotide synthesis using* [12].

Multiple DNP phosphoramidite **[12]** which was readily soluble in anhydrous acetonitrile, was used as a 0.15M solution for oligonucleotide synthesis. The coupling efficiency was found to be 97.5% when **[12]** was used with a standard 0.2μmol or 1μmol scale DNA synthesis cycle. This could be improved to 98.5% by increasing the wait time for the coupling reaction to 2 minutes. Oligonucleotides labelled at the 3'-end were synthesized by coupling one or more additions of **[12]** onto normal T solid support followed by the synthesis of the desired oligonucleotide.

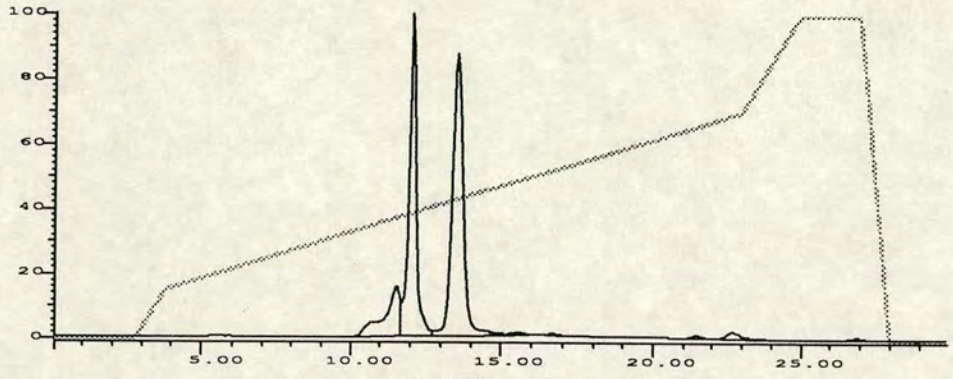
6.3. *HPLC analysis and purification of oligonucleotides DNP-labelled using* [12].

As only one DNP group is introduced per synthesis cycle when using **[12]**, the lipophilicity of DNP oligonucleotides labelled with **[12]** is less than that of oligonucleotides labelled with the same number of additions of **[3]**. However the DNP oligonucleotides were still found to elute on HPLC several minutes later than unlabelled failure sequences. The full HPLC conditions for purification of DNP-labelled oligonucleotides are given in the experimental section. A suitable buffer system for use with all DNP labelled oligonucleotides (either Trityl On, or Trityl Off syntheses) was found to be a gradient of 0-50% acetonitrile in 0.1M ammonium acetate.

6.4. *Stability of the DNP label to DNA Synthesis and Deprotection Conditions.*

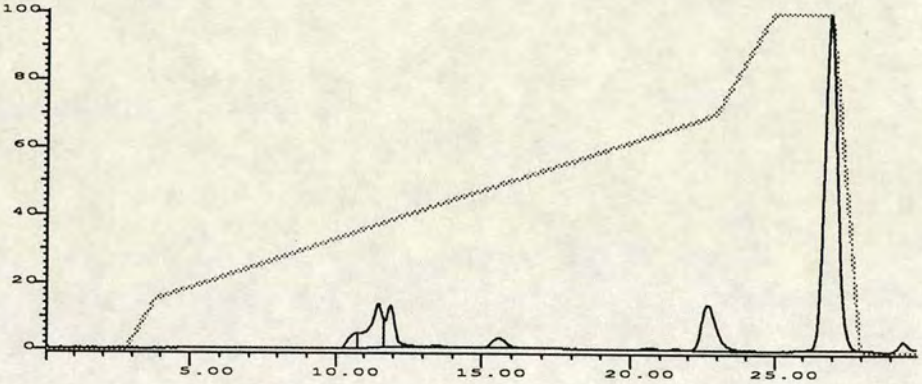
Misiura *et al.* [27] reported that labelled oligonucleotides synthesized using the 3-aminopropyl solketal backbone had to be synthesized with the 5'-terminal dimethoxytrityl group in place (Trityl-On synthesis). This was necessary to prevent attack of the free terminal primary hydroxyl function on the adjacent phosphodiester linkage under the strongly basic conditions used for oligonucleotide deprotection. As **[12]** contains the same vicinal diol arrangement found in the 3-aminopropyl solketal backbone it was thought necessary to confirm this finding by synthesizing the test sequence **[12]**pTpTpTpTpT both Trityl On and Trityl Off and monitoring any degradation in aqueous ammonia by HPLC. *Figure 25* shows the HPLC chromatograms of (a) Trityl Off and (b) Trityl On test sequence after heating at 55°C in NH_4OH for 2 hours. As expected the Trityl Off synthesis resulted in degradation of the label. Peak enhancement injections with pure TpTpTpTpT confirmed that the result of the degradation was loss of the labelling group to form the underivatized oligonucleotide (*Figure 25*, Peak (I)). There was no loss of label for the Trityl Off synthesis of internally labelled Tp**[12]**pTpTpTpTpT, in which both hydroxyl functions of the vicinal diol are 'protected' by the phosphodiester linkage. The probable mechanism of degradation is shown in *Figure 26*. This problem is easily overcome by synthesizing oligonucleotides Trityl On and the presence of the very lipophilic 5'-terminal dimethoxytrityl group makes the purification of the DNP-labelled oligonucleotide from its failure sequences even easier.

As with the single addition DNP phosphoramidite **[5]**, the DNP group of **[12]** was found to be stable to ammonia deprotection conditions.



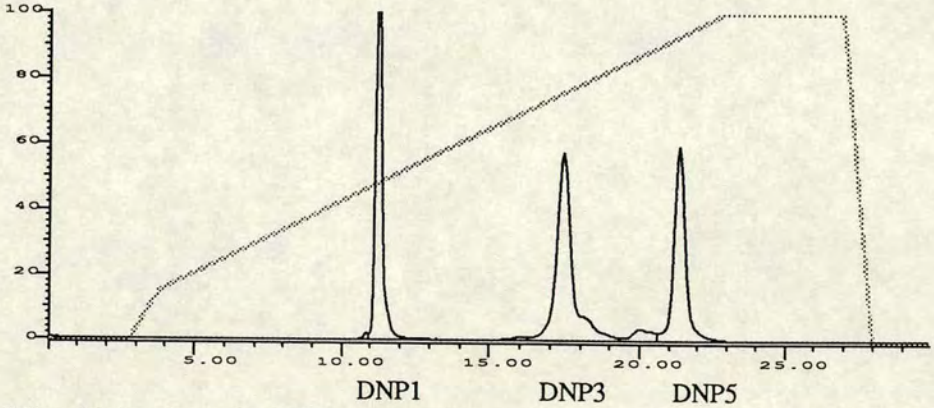
(I) (II)

(a) [**12**]pTpTpTpTpT, Trityl Off after 2 hours in NH_4OH at 55°C.
(I) Degradation product TpTpTpTpT. (II) Undegraded [**12**]pTpTpTpTpT.



(I)

(b) [**12**]pTpTpTpTpT , Trityl On after 2 hours in NH_4OH at 55°C.
(I) Trityl On [**12**]pTpTpTpTpT



(c) Mixed injection of a PCR primer (24mer) labelled with 1,3 and 5 DNP groups (using [**12**]), after trityl on purification and subsequent detritylation.

Figure 25: HPLC chromatograms of DNP labelled oligonucleotides.

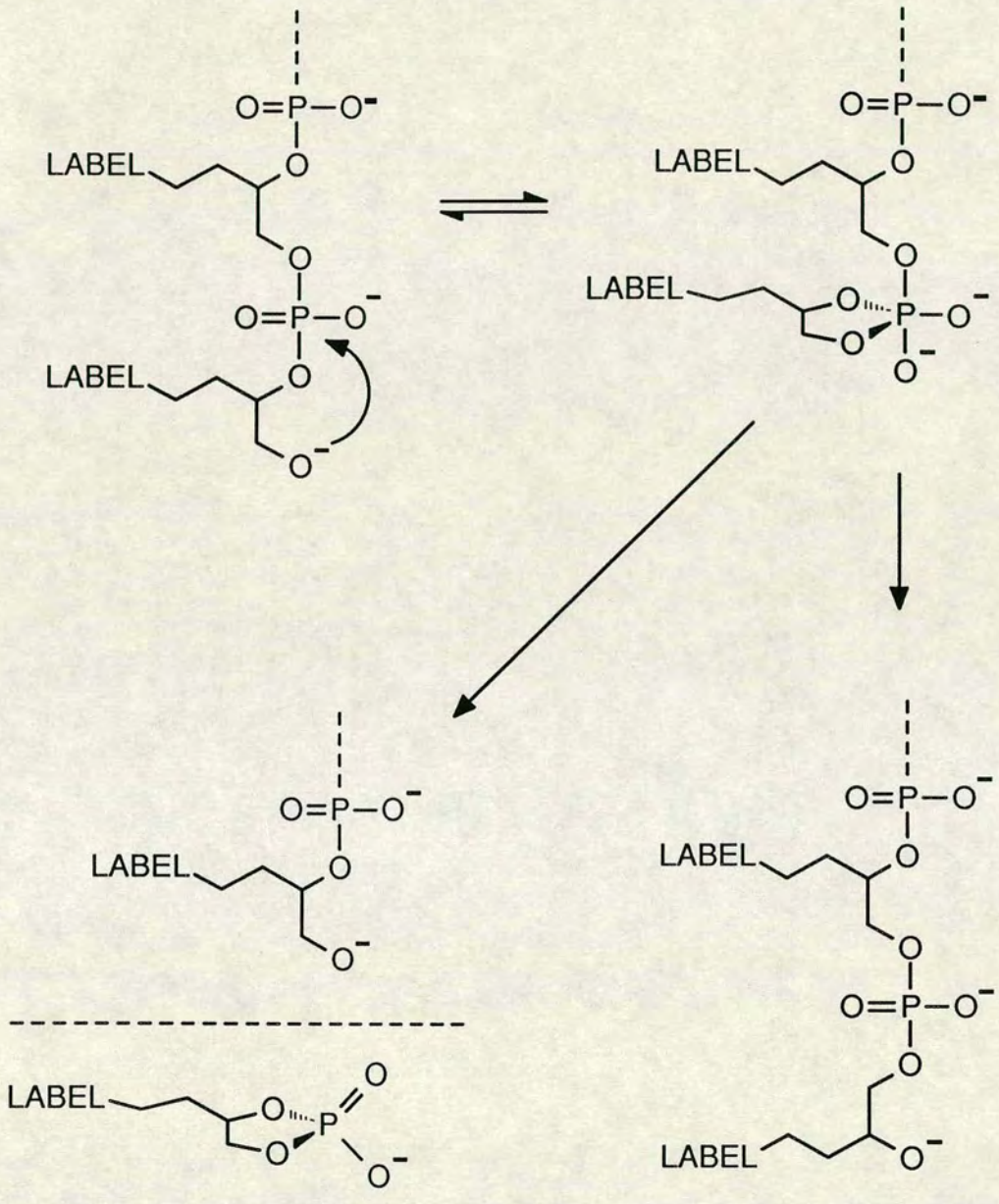


Figure 26: Possible mechanism of degradation of 1,2-diol-linked labels under ammonia deprotection conditions.

Figure 25c shows HPLC chromatograms of 24mer oligonucleotides (GW1; Appendix 1) labelled with 1, 3 and 5 DNP groups. Increasing the number of DNP groups attached to the oligonucleotide causes the oligonucleotide to elute later on reversed-phase HPLC.

6.5. *Synthesis of Hybridisation Probes and PCR primers.*

Probes (40mer; HC1; Appendix 1) for use in *in situ* hybridisation experiments for the detection of Epstein-Barr virus in clinical samples were synthesized with 1,3 and 5 DNP groups. Epstein-Barr virus causes mononucleosis (glandular fever), and has been linked with Burkitt's lymphoma and nasopharyngeal carcinoma, two common forms of cancer in Africa and China.[102] These oligonucleotides have been supplied to Dr. Heather Cubie at the Regional Virology Laboratory at the City Hospital in Edinburgh. The detection limits of these DNP labelled probes will be compared to those of more expensive Digoxigenin labelled probes.

6.6. *Antibody Purification.*

In order to allow the capture of DNP oligonucleotides or PCR products with an *anti*-DNP antibody immobilised on a solid support it is essential that the antibody is pure and free from plasma proteins. In order to allow such experiments to be carried out a pure sample of K3 *anti*-DNP antibody was obtained by Protein A column chromatography of ascitic fluid using a commercially available kit (Pierce). The ascitic fluid was found to have an antibody concentration of approximately 7mg ml^{-1} as measured spectrophotometrically at 280nm. The antibody was assumed to have an extinction coefficient at 280nm of 1.45L g^{-1} cm. [103] The *anti*-DNP activity of the purified antibody was confirmed by the

detection of DNP oligonucleotides which were fixed by UV irradiation onto nylon filters. Samples of the purified antibody have been supplied to collaborators for use in 'Capture and Detect' experiments.

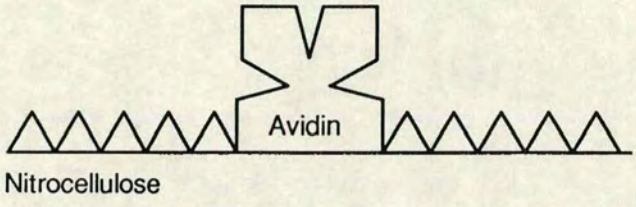
6.7. *Capture and Detect Experiments on Biotinylated DNP Oligonucleotides.*

In a preliminary experiment to determine the feasibility of the capture of PCR products containing both DNP groups and biotin on immobilised avidin, oligonucleotides (24mer PCR primers; GW1; Appendix 1) were labelled with 1 or 3 DNP groups at the 3'-end and a single biotin moiety (Bio) at the 5'-end.[87] The experimental design is summarised in *Figure 27.* A solution of avidin was spotted onto a nitrocellulose filter to give a 0.1μg loading per spot. After incubation at 4°C for 4 hours the filter was blocked with a 10% solution of fat-free skimmed milk in phosphate-buffered saline (PBS). The filter was then cut into strips and each strip was incubated with a 0.175μM solution of different 24mer oligonucleotides.

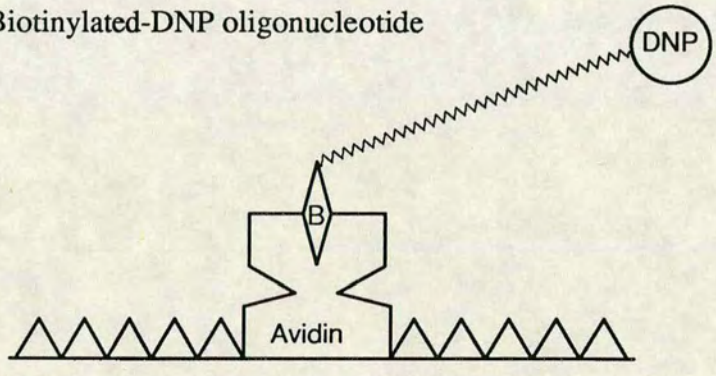
The strips were washed and incubated with K3 *anti*-DNP antibody followed by goat-*anti*-mouse IgG-HRP conjugate. The strips were then incubated with diaminobenzidine (DAB) to give the colour reaction. The results are shown in *Figure 28(a)* . The experiment has clearly worked well. The increased signal from the $^{5'}$Bio----------DNP$_3$$^{3'}$ oligonucleotide compared with the $^{5'}$Bio----------DNP$_1$$^{3'}$ oligonucleotide indicates that multiple DNP groups do indeed give an enhanced signal compared to single DNP groups. One minor problem was the appearance of a faint background signal at the positions where avidin was spotted in the negative control experiments. This signal only appeared after prolonged incubation with DAB and was present even in the strip where no

(1) Immobilise avidin on nitrocellulose

(2) Block membrane with inert protein



(3) Incubate with Biotinylated-DNP oligonucleotide



(3) Incubate with anti-DNP antibody, then secondary antibody-enzyme conjugate

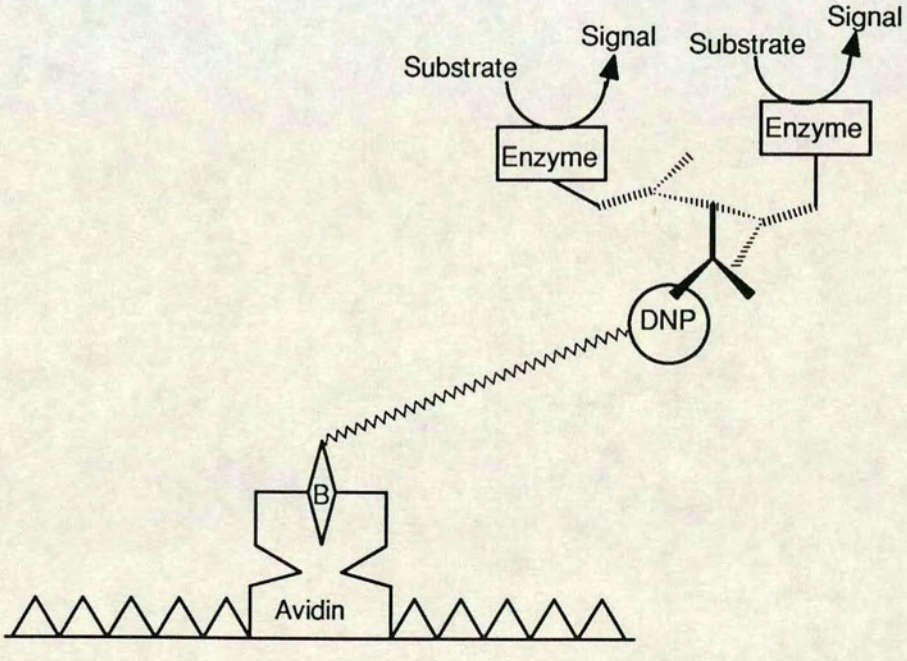


Figure 27: Capture and Detection of a biotinylated DNP oligonucleotide

oligonucleotide was added. The most likely cause of this signal is the presence in the polyclonal mixture which comprises the goat-*anti*-mouse IgG-HRP conjugate of an antibody which binds weakly to avidin. This slight problem could probably be overcome by the use of streptavidin which should exhibit fewer non-specific interactions.

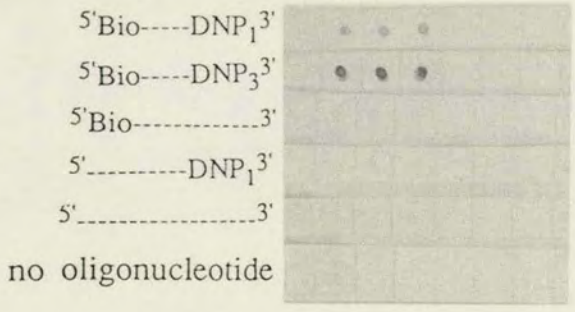
These experiments have proven the feasibility of carrying out capture and detect experiments on biotin/DNP PCR products. Such experiments are now being carried out by collaborators.

6.8. *Comparison of the Detection Sensitivity of DNP and Biotinylated Oligonucleotides.*

In order to compare the sensitivity of detection of oligonucleotides labelled with different numbers of DNP groups with biotinylated oligonucleotides a simple assay was developed. The DNP or biotin labelled oligonucleotides (GW1, Appendix 1) were immobilised on nylon membranes by UV irradiation for 5 minutes. The DNP groups were detected by incubation with K3 antibody followed by secondary antibody-HRP conjugate. Biotin was detected by incubation with avidin-HRP conjugate and colour development was carried out with DAB. In order to ensure that the presence of multiple DNP groups did not affect the binding of the oligonucleotides to the nylon membrane, oligonucleotides containing both biotin and DNP groups were synthesized and fixed to nylon. If multiple DNP groups increased oligonucleotide binding then an enhanced signal would be expected for detection of the biotin residue at the other end of the oligonucleotide. The experiments were carried out as follows:

Five-fold dilutions of the labelled 24mer oligonucleotides were spotted and fixed in duplicate onto nylon membranes starting at 1pmol

(a) Results of Capture and Detection of oligonucleotides (using Avidin on nitrocellulose).



(b) Detection of Oligonucleotides fixed on nylon.

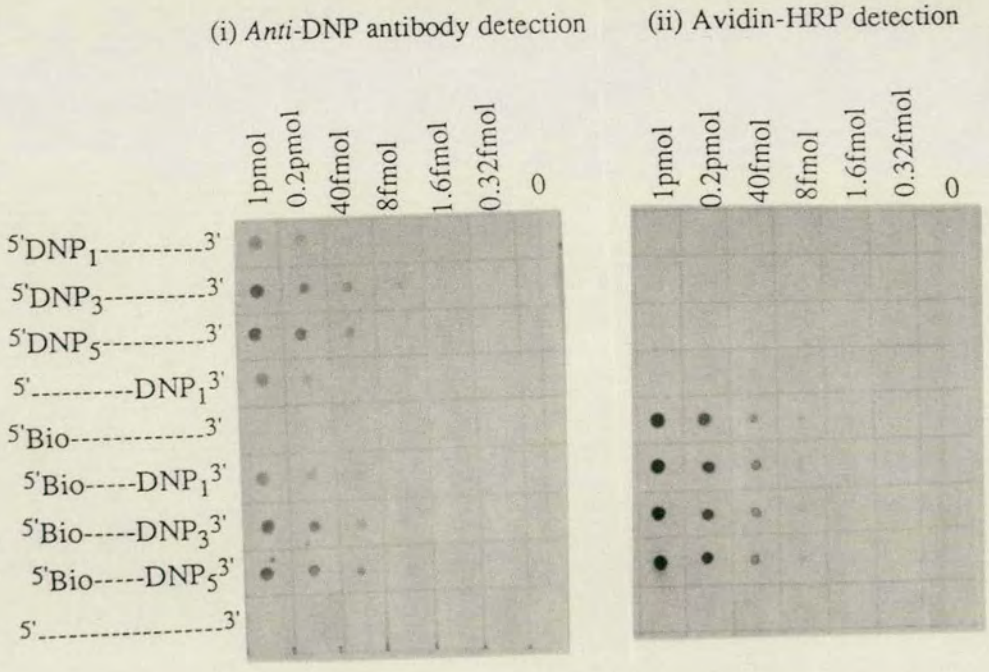
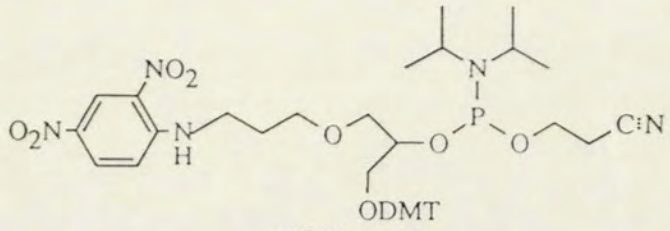
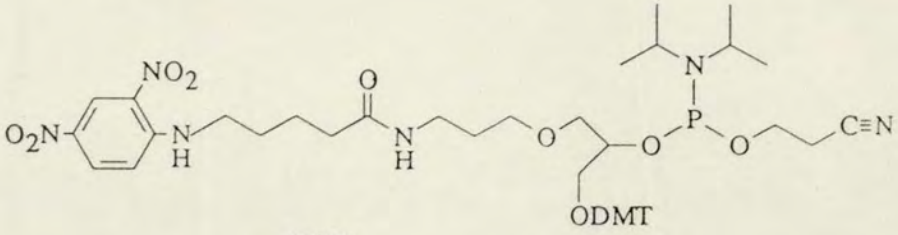


Figure 28

(a) DNP phosphoramidites of John Grzybowski.

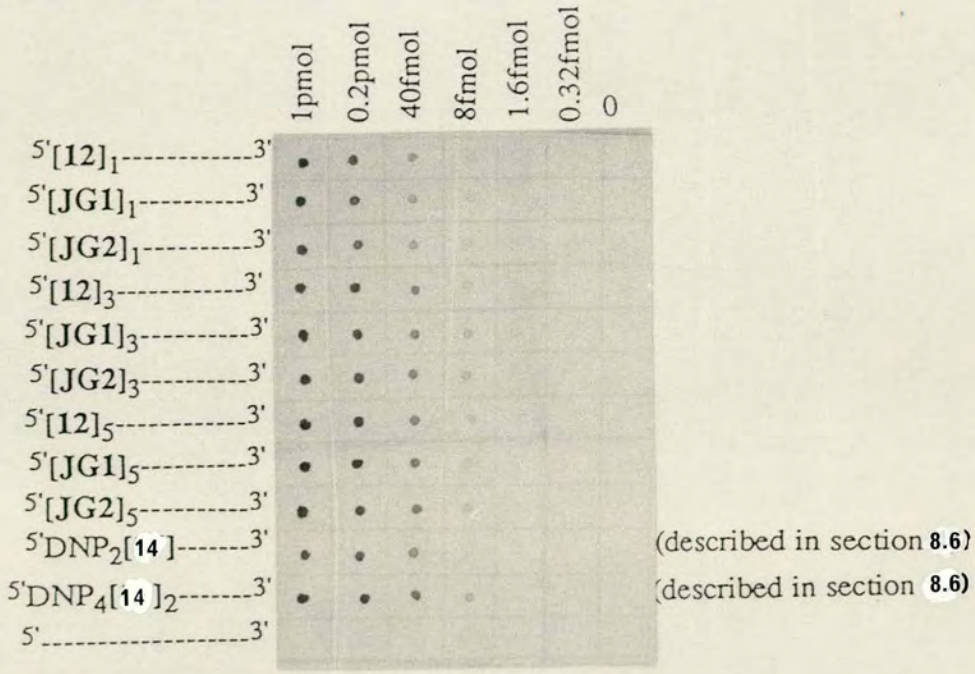


[JG1]



[JG2]

(b) Detection of oligonucleotides with different spacer arms fixed on nylon (using *anti*-DNP antibody/ secondary antibody-HRP conjugate).



(described in section 8.6)

(described in section 8.6)

Figure 29

loading. After blocking with non-fat skimmed milk solution, one membrane was incubated with avidin-HRP conjugate, and the other with K3 antibody followed by goat-*anti*-mouse IgG antibody-HRP conjugate. Both membranes were then developed with DAB. The results of this experiment are shown in *Figure 28(b)*

Conclusions:

(i) There is a five-fold increase in sensitivity between the DNP_1 the DNP_3 labelled oligonucleotides. There is no increase in sensitivity between the DNP_3 and DNP_5 labelled oligonucleotides. This would suggest that 3 DNP groups is the optimum number to maximise the sensitivity:cost ratio. The reason for the increase in sensitivity is unlikely to be due to the binding of more than one antibody as the antibody is much larger than both the labelling groups and the oligonucleotide. The distance between the two hapten binding sites of an IgG is approximately 200A.[104] An increase in antibody binding affinity due to the presence of multiple haptens is a more likely explanation.

(ii) There is no difference in sensitivity between oligonucleotides labelled at their 3'- and 5'-ends with a single DNP group.

(iii) The presence of the DNP groups does not affect the binding of the oligonucleotides to the nylon as the biotin signal is identical for oligonucleotides with and without DNP groups.

(iv) DNP_3 and DNP_5 labelled oligonucleotides give comparable sensitivity to biotinylated oligonucleotides. The limit of detection using this simple assay was 8 fmol of oligonucleotide.

6.9. *Effect of length of spacer arm on sensitivity of detection.*

Two DNP phosphoramidites based on the backbone of Misiura *et al.* were synthesized and used by John Grzybowski of this group to synthesize DNP oligonucleotides. These two phosphoramidites are shown in *Figure 29(a)* . It was thought that the longer spacer arms of **[JG1]** and especially **[JG2]** would enhance the accessibility of the DNP groups to the antibody and minimise steric crowding. The same assay conditions as described above (Section 6.8.) were used. The results of this experiment are shown in *Figure 29(b).* (Sequence GW1, Appendix 1)

Conclusion: The length of the spacer arm has no effect on the sensitivity of detection in this assay system.

7.0. Overall Conclusions on DNP phosphoramidites.

(i) The DNP phosphoramidite **[12]** is easy to synthesize using simple reactions which are amenable to scale-up.

(ii) Single or multiple DNP groups can be incorporated into oligonucleotides at the 3'- or 5'-end during standard DNA synthesis conditions.

(iii) Trityl On synthesis is essential for 5'-end labelling with **[12]**.

(iv) The DNP oligonucleotides are readily purified by reverse-phase HPLC due to their lipophilicity.

(v) The DNP label is suitable for use in 'Capture and Detect' experiments.

(vi) Three DNP groups is the optimum number for detection.

(vii) The length of the spacer arm connecting the DNP group to the rest of the backbone has no effect on sensitivity under the assay conditions used.

8.0. Synthesis and Uses of a Multiple Addition Hydroxyl Phosphoramidite.

The introduction of multiple hydroxyl functions to the 5'-end of oligonucleotides during solid-phase synthesis and the subsequent coupling of a simple single addition labelling phosphoramidite should allow the attachment of multiple labelling groups to the oligonucleotide. This circumvents the need for the lengthy synthetic routes necessary for the synthesis of multiple addition labelling phosphoramidites.

The introduction of multiple hydroxyl functions into oligonucleotides has been carried out previously using a modified nucleoside phosphoramidite.[105,106] and also a glycerol phosphoramidite.[107] The aim in those studies was to synthesize 'fork and comb-shaped' oligonucleotides. Hybridisation to a target sequence by the 'handle' of the fork or comb, and subsequent hybridisation of multiple labelled oligonucleotides to the 'teeth' of the fork or comb, allowed the detection of very low levels of the target sequence.

8.1. *Chemical Synthesis.*

The simplest method of introducing multiple hydroxyl functions is *via* a protected triol phosphoramidite. This approach was used by Horn *et al.* to synthesize branched oligonucleotides. In that study glycerol was used as a triol starting material. The multiple hydroxyl phosphoramidite was not used for multiple non-radioactive labelling of oligonucleotides.

A suitable triol starting material for a multiple hydroxyl phosphoramidite is 1,2,6-trihydroxyhexane. Although this molecule is asymmetric, the tetramethylene spacer between one of the primary hydroxyl functions and the backbone is likely to improve both the coupling characteristics of the phosphoramidite, and the antibody or

streptavidin mediated recognition of labelling groups attached to it. The synthetic route is outlined in *Scheme 4*. The two primary hydroxyl functions were selectively protected as their 4, 4'-dimethoxytrityl ethers, by reaction with 4, 4'-dimethoxytrityl chloride in pyridine to give compound **[13]**. Subsequent phosphitylation of the secondary hydroxyl function proceeded smoothly using 2-cyanoethyl N,N-diisopropyl-phosphoramidochloridite in the presence of N,N-diisopropylethylamine, to give **[14]** as an oil.

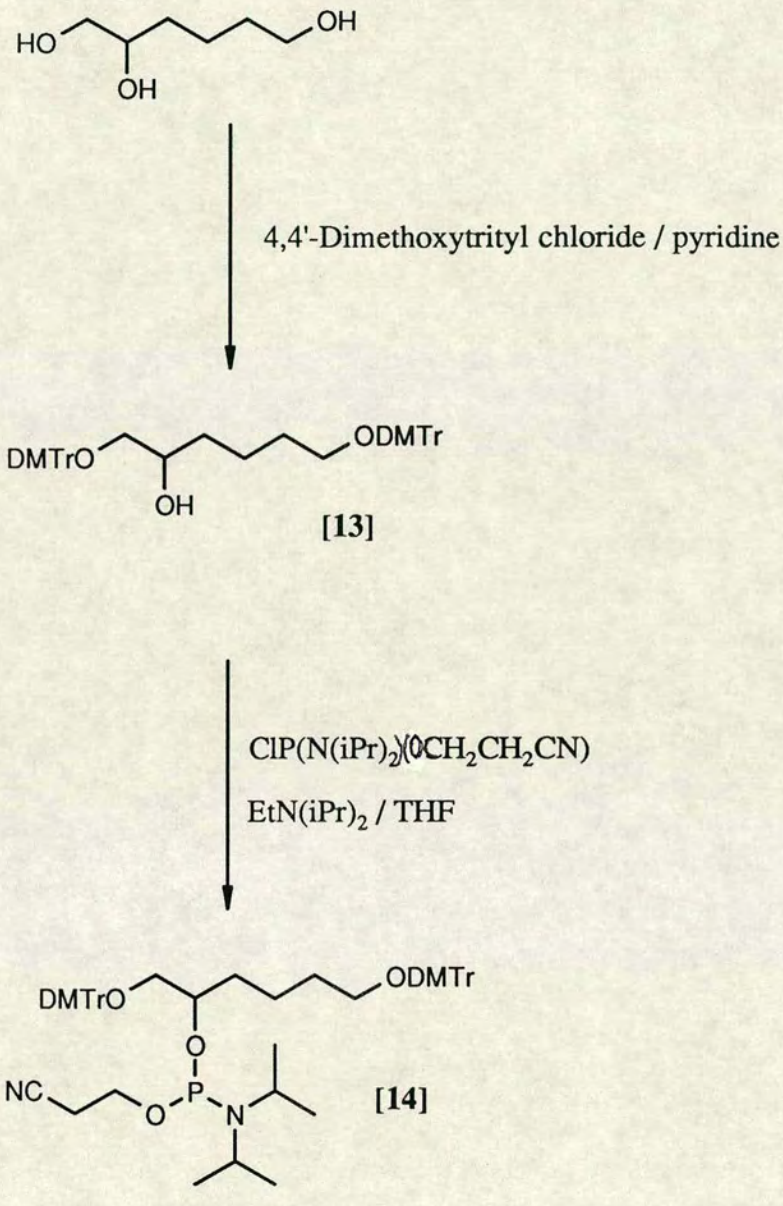
8.2. *Oligonucleotide Synthesis using multiple hydroxyl phosphoramidite* [14].

8.2.1. *Solubility.*

In spite of its high lipophilicity, **[14]** was found to be soluble in anhydrous acetonitrile and was used as a 0.15M solution in this solvent for DNA synthesis.

8.2.2. *Coupling Efficiency.*

The coupling of n additions of **[14]** results in the introduction of 2^n hydroxyl functions onto the 5'-end of the oligonucleotide. For example, this means that the coupling of 2 additions of **[14]** during a 0.2μmol synthesis introduces 0.8μmol of hydroxyl functions. These hydroxyl functions are then available for the coupling of any phosphoramidite (*eg.* a biotin phosphoramidite; single addition DNP phosphoramidite **[5]**; further additions of **[14]**; or normal DNA synthesis monomers). During a normal 0.2μmol scale synthesis the phosphoramidite is added in a 10-fold excess over the solid-support bound 5'-hydroxyl functions. The introduction of 4 hydroxyl functions effectively changes a 0.2μmol scale synthesis into a 0.8μmol scale synthesis and the normal phosphoramidite delivery would then give only a 2.5-fold excess of phosphoramidite. This


HO
OH
OH
4,4'-Dimethoxytrityl chloride / pyridine
DMTrO
ODMTr
OH
[13]
ClP(N(iPr)2)(OCH2CH2CN)
EtN(iPr)2 / THF
DMTrO
ODMTr
O
NC
O
P
N
[14]

Scheme 4

has repercussions on the kinetics and efficiency of the coupling reaction. It was thus necessary to modify the synthesis cycle to allow a double delivery of phosphoramidite + tetrazole for the coupling of **[14]** and the subsequent labelling phosphoramidite. This double delivery of phosphoramidite does not fully compensate for the increase in number of μmoles of primary hydroxyl groups bound to the solid support, so a 5 minute wait time for the coupling of **[14]** and the subsequent labelling phosphoramidite was also included in the synthesis cycle. A schematic representation of the sequence of events using **[14]** and **[5]** for multiple DNP labelling is shown in *Figure 30*.

In order to test coupling efficiencies a sequence $^{5'}$Bio**[14][14]**pTpTpTpTpT$^{3'}$ was synthesized. After two successive couplings of **[14]** to the 5'-end of the oligonucleotide the biotin residues were introduced by a single coupling with the biotin phosphoramidite of Pon.[87] This biotin phosphoramidite contains a dimethoxytrityl group and so its coupling efficiency can be measured. During detritylation the amount of dimethoxytrityl cation released doubles for every addition of **[14]**. The coupling efficiencies were therefore corrected to allow for this effect. The coupling efficiencies for the test sequence under different synthesis conditions are shown below.

		Coupling efficiency		
Coupling	Coupling wait	**[14]**-T	**[14]**-**[14]**	Bio-**[14]**
Normal	30 seconds	83%	86%	70%
Normal	5 minutes	91%	90%	75%
Double delivery	5 minutes	91%	89%	92%

Although the coupling efficiencies are not >98.5% typical of normal DNA synthesis, they are high enough for reasonable yields of the desired product. The 92% coupling efficiency of the biotin

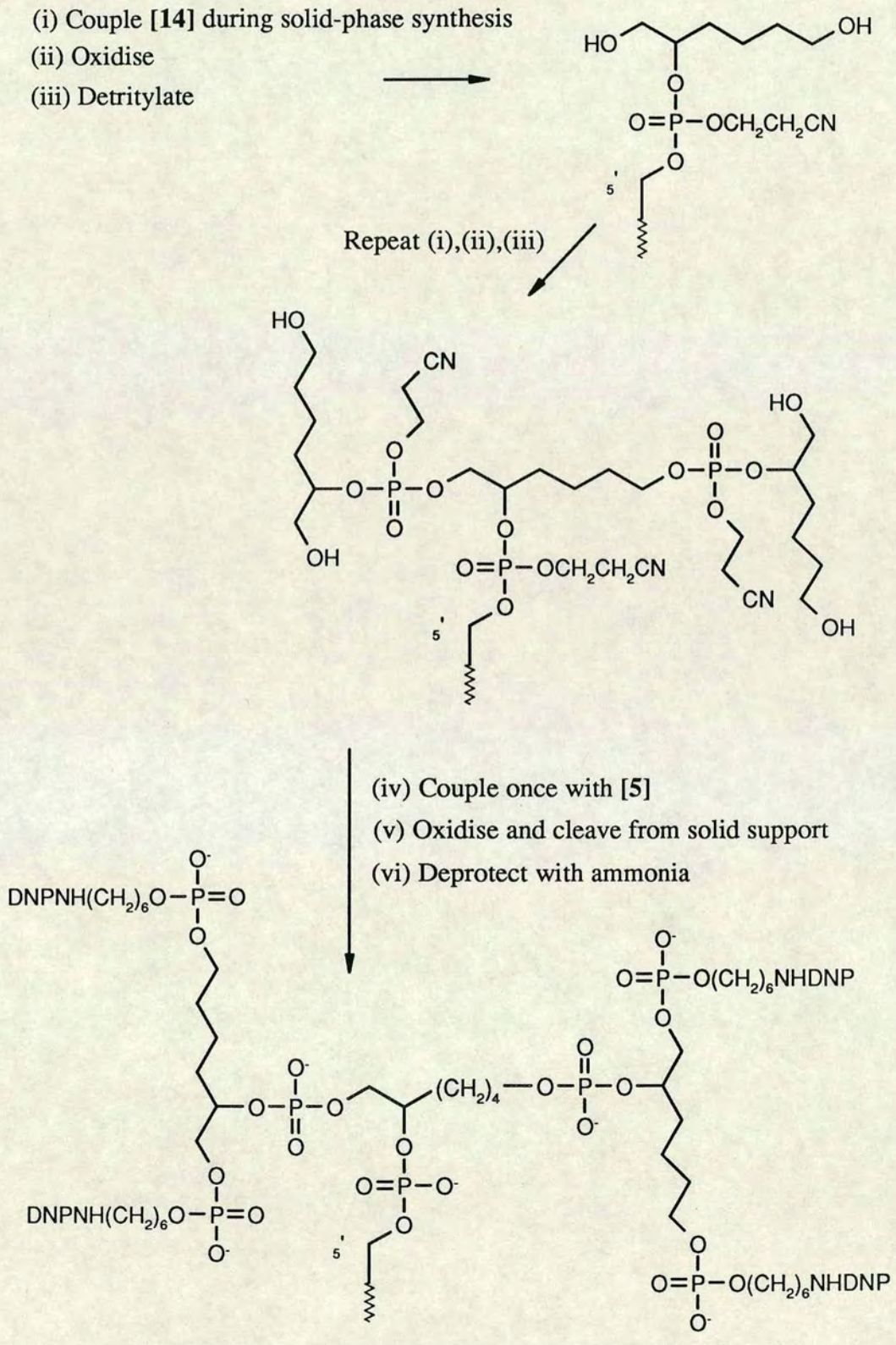
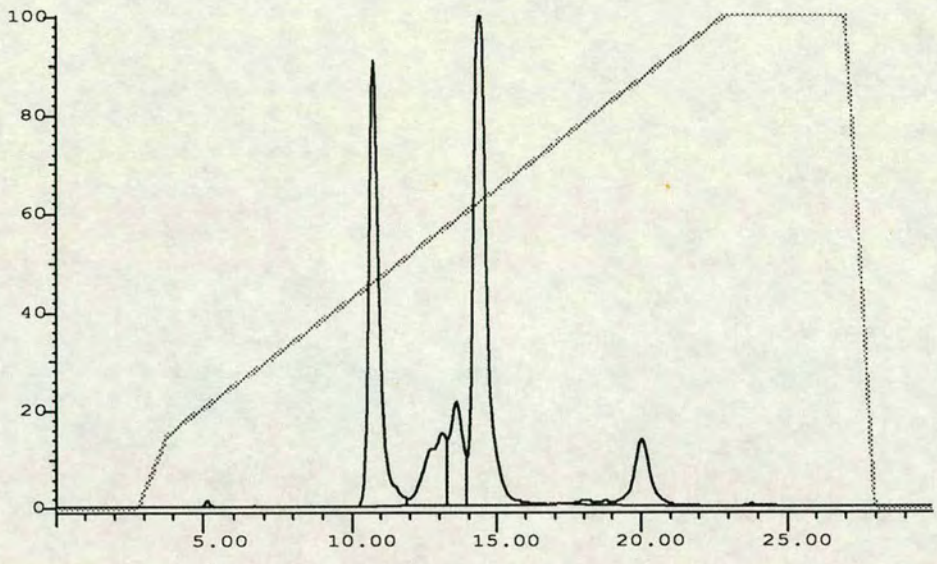


Figure 30: Multiple DNP labelling using [14] and [5].

phosphoramidite to the multiple hydroxyl functions means that only a very small proportion of the multiple-hydroxyl oligonucleotides will have no biotins attached. The coupling efficiency of the biotin phosphoramidite under normal DNA synthesis conditions was 97%. The synthesis cycle incorporating the double delivery of phosphoramidite + tetrazole and the 5 min wait step for coupling was used in all subsequent syntheses using **[14]**.

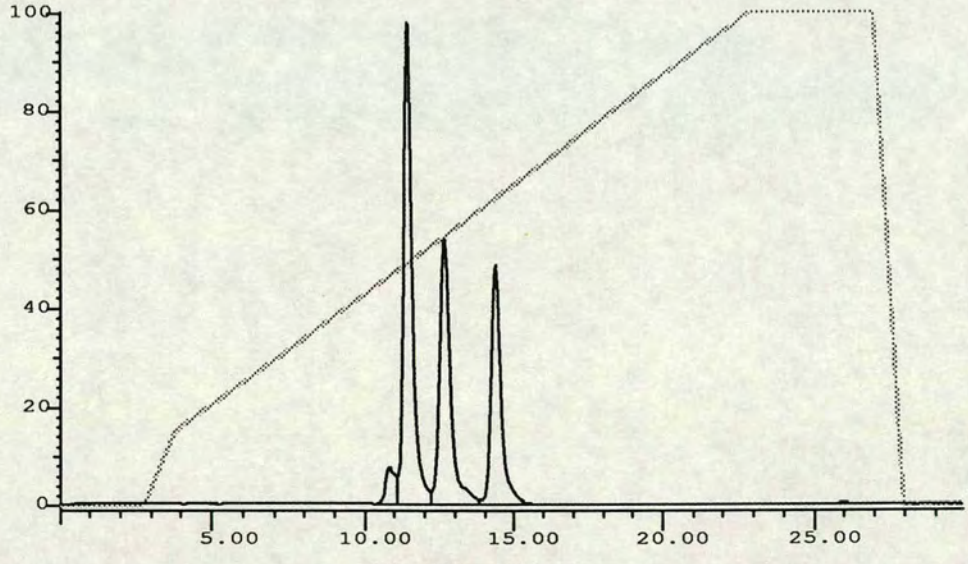
8.3. *Synthesis and Purification of Multiply Biotinylated Oligonucleotides using* [14].

PCR primers (24mers, GW1, Appendix1) were synthesized with either 1 or 2 additions of **[14]** at the 5'-end, followed by a single coupling of biotin phosphoramidite. This gave primers with either 2 or 4 biotin residues at the 5'-end. The HPLC chromatogram of crude tetrabiotinylated primer is shown in *Figure 31a.* The first peak to elute (I) is unbiotinylated failure sequences. The smaller peaks (II) contain either 1, 2 or 3 biotins. The presence of biotin in these oligonucleotides was confirmed by spraying with *p*-dimethylamino-cinnamaldehyde solution.[108,109] The desired product is the second large peak to elute (III), and is obviously well separated from unlabelled failure sequences. The identity of peak (IV) is not known. The HPLC chromatogram of crude di-biotinylated primer was very similar, however the separation between failure sequences and product was smaller. The HPLC chromatogram of a mixed injection of the same purified PCR primer bearing 1, 2, and 4 biotins is shown in *Figure 31b* to illustrate the increase in lipophilicity as the number of biotin residues is increased.



(I) (II) (III) (IV)

(a) HPLC chromatogram of a crude 24mer oligonucleotide tetrabiotinylated using **[14]** and a biotin phosphoramidite.



Bio Bio_2 Bio_4

(b) Mixed injection of a 24mer oligonucleotide bearing 1, 2 and 4 biotin groups.

Figure 31

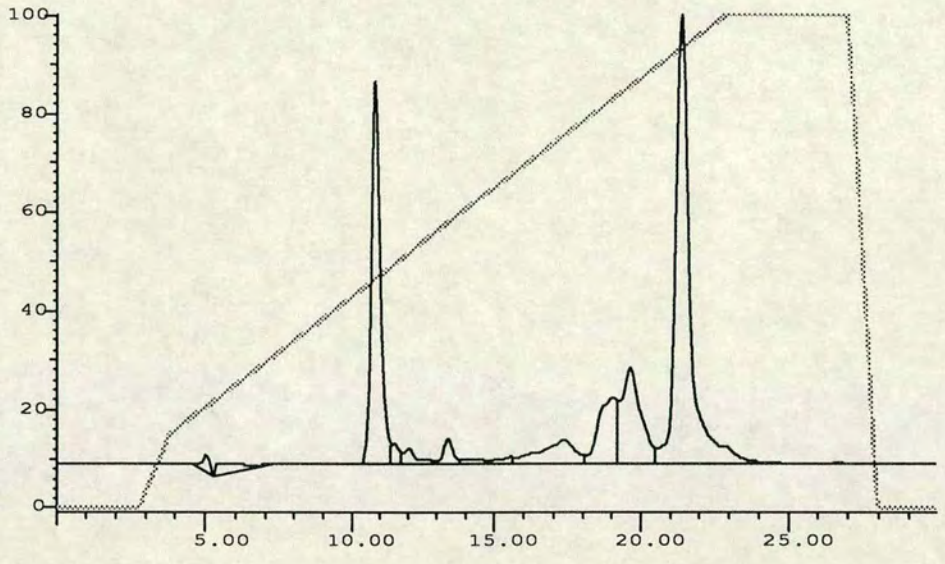
8.4. *Synthesis and Purification of Multiple DNP Oligonucleotides using* [14] *and* [5].

The same procedure as described in section 8.3. above was used for the synthesis of multiple DNP oligonucleotides (24mer, GW1, Appendix 1), however DNP phosphoramidite **[5]** as a 0.15M solution in acetonitrile was used instead of biotin phosphoramidite. The HPLC chromatogram of crude DNP_4 primer is shown in *Figure 32a.* Peak (I) is a mixture of failure sequences. The smaller peaks (II) are oligonucleotides with 1,2 and 3 DNP groups, and are yellow. The extremely lipophilic product is the last peak to elute (III), and is bright yellow. A mixed injection of the same PCR primer bearing 1, 2, and 4 DNP groups is shown in *Figure 32b* to illustrate the increase in lipophilicity as the number of DNP groups is increased. Probes for the detection of Epstein-Barr virus were synthesized (40mer, HC1, Appendix 1) with 2 and 4 DNP groups using **[14]** and **[5]** and are now being evaluated by collaborators.

8.5. *Detection of Multiply Biotinylated Oligonucleotides.*

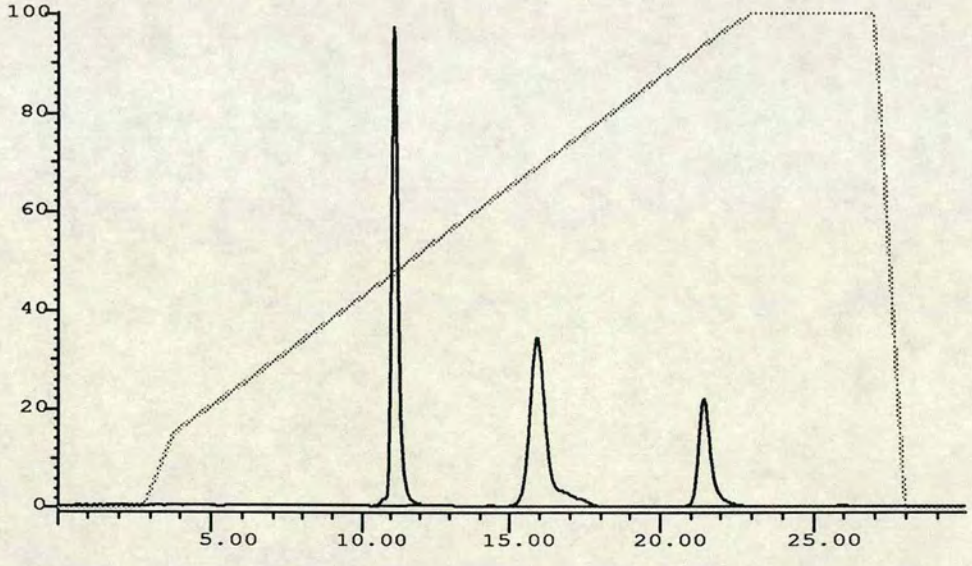
To determine if the attachment of multiple biotin moieties to oligonucleotides using **[14]** gave enhanced detection sensitivity, a series of three-fold dilutions of oligonucleotides (24mer, GW1, Appendix 1) bearing 1, 2, and 4 biotins were spotted onto nylon and fixed by UV irradiation. The biotin was detected using avidin-HRP conjugate followed by colour development with DAB.

The results of this experiment are shown in *Figure 33a.* It is clear that there is only slight enhancement in sensitivity using multiply biotinylated oligonucleotides and an avidin-based detection system. This is similar to the result reported by Misiura *et al.* [27] (Section 3.2.2.).



(I) (II) (III)

(a) HPLC chromatogram of a crude 24mer oligonucleotide bearing 4 DNP groups, labelled using **[14]** and **[5]**.

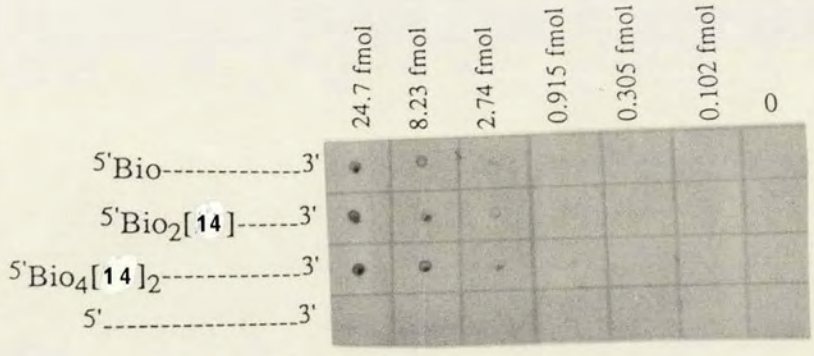


DNP_1 DNP_2 DNP_4

(b) Mixed injection of a 24mer oligonucleotide bearing 1, 2 and 4 DNP groups, labelled using **[14]** and **[5]**.

Figure 32

(a) Detection of biotinylated oligonucleotides fixed on nylon (using Avidin-HRP conjugate).



(b) Detection of oligonucleotides fixed on nylon (using *anti*-DNP antibody/ secondary antibody-HRP conjugate).

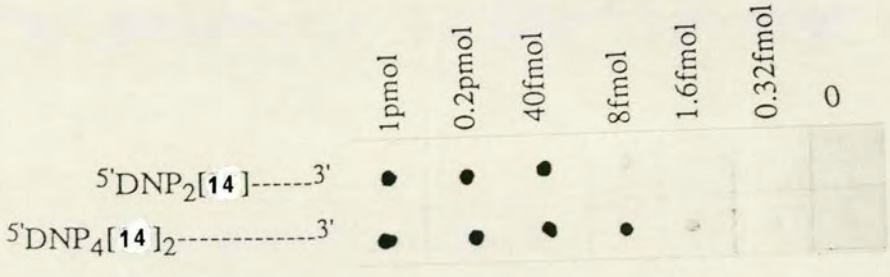


Figure 33

Detection using *anti*-biotin antibodies has not yet been attempted, however a detection system using avidin or streptavidin is preferable due to the commercial availability of a wide variety of derivatives of these proteins and the high affinity they have for biotin.

8.6. *Detection of Multiple DNP Oligonucleotides.*

The same procedure as that in section 6.8. above was carried out using five-fold dilutions of oligonucleotides (24mer, GW1, Appendix 1) labelled with 2 and 4 DNP groups using **[14]** and **[5]** to compare with oligonucleotides labelled with a single DNP group using **[12]**. The results are shown in *Figure 33b* .

The oligonucleotide labelled with 2 DNP groups (using**[14]** and **[5]**) gives comparable sensitivity to those labelled with a single DNP group (using **[5]**). The oligonucleotide labelled with 4 DNP groups (using**[14]** and **[5]**) gives comparable sensitivity to those labelled with 3 or 5 DNP groups using multiple addition DNP phosphoramidite **[12]**. (*Figure 29*).

8.7. *Conclusions.*

(i) The chemical synthesis of multiple hydroxyl phosphoramidite **[14]** is readily achieved in 2 steps from an inexpensive starting material.
(ii) The phosphoramidite **[14]** is soluble in acetonitrile, the solvent of choice for DNA synthesis.
(iii) The introduction of multiple hydroxyl functions onto the 5'-end of oligonucleotides during solid-phase synthesis is readily achieved in high yield using **[14]**.

(iv) The use of a slightly modified synthesis cycle is necessary to increase the yield of the product. These minor modifications do not add significantly to the time and cost of DNA synthesis.
(v) Multiple labelling groups can be attached in high yield to oligonucleotides by a single coupling reaction after the desired number of additions of **[14]**.
(vi) Reversed-phase HPLC purification of the labelled oligonucleotides is easy due to their enhanced lipophilicity.
(vii) There is little (<3-fold) enhancement in sensitivity on labelling with multiple biotins compared to single biotin labelling when avidin detection is used.
(viii) An approximately five-fold increase in sensitivity is observed for labelling with 4 DNP groups compared to single DNP labelling.

8.8. *Work in Progress and Future Work.*

8.8.1. *Synthesis of Branched Oligonucleotides.*

The simultaneous synthesis of two identical oligonucleotides joined at the 3'-ends may produce oligonucleotides with interesting hybridisation properties, as a complementary strand would be antiparallel to both strands of the branched oligonucleotide on hybridisation. This work is being carried out at present.

8.8.2. *Synthesis of RNA with multiple biotin labels.*

Multiple hydroxyl phosphoramidite **[14]** has been used successfully by Susanne Ebel of this research group for the synthesis of oligoribonucleotides carrying four biotin residues. These are being used by collaborative researchers for the affinity purification of small nuclear ribonucleoproteins.

9.0. Synthesis of Benzoyl Protected Biotin Phosphoramidites.

In spite of its disadvantages, biotin remains the non-radioactive label of choice for oligonucleotides in many biological and medical applications. The traditional method for biotinylation of oligonucleotides, by reaction of biotin-N-hydroxysuccinimide ester with an amino modified oligonucleotide has largely been superceded by the use of biotin phosphoramidite monomers. These allow rapid one-step automated biotinylation during solid-phase synthesis. Some biotin phosphoramidites, designed to introduce single or multiple biotins to the 5'-end of oligonucleotides (*Figure 12*)[27,85,86] have the disadvantages of a short spacer arm[85] or an unprotected biotin ring[27,86] which has been shown to lead to side-reactions during oligonucleotide synthesis.[93] Other phosphoramidites, based on modified nucleosides[91,93] (*Figure 13*) allow biotinylation at any position but require lengthy and expensive syntheses. The phosphoramidite of Pon[87] has none of the above disadvantages but uses the rather bulky and relatively expensive dimethoxytrityl group for protection of the biotin ring. This phosphoramidite became commercially available as the work described below was being carried out.

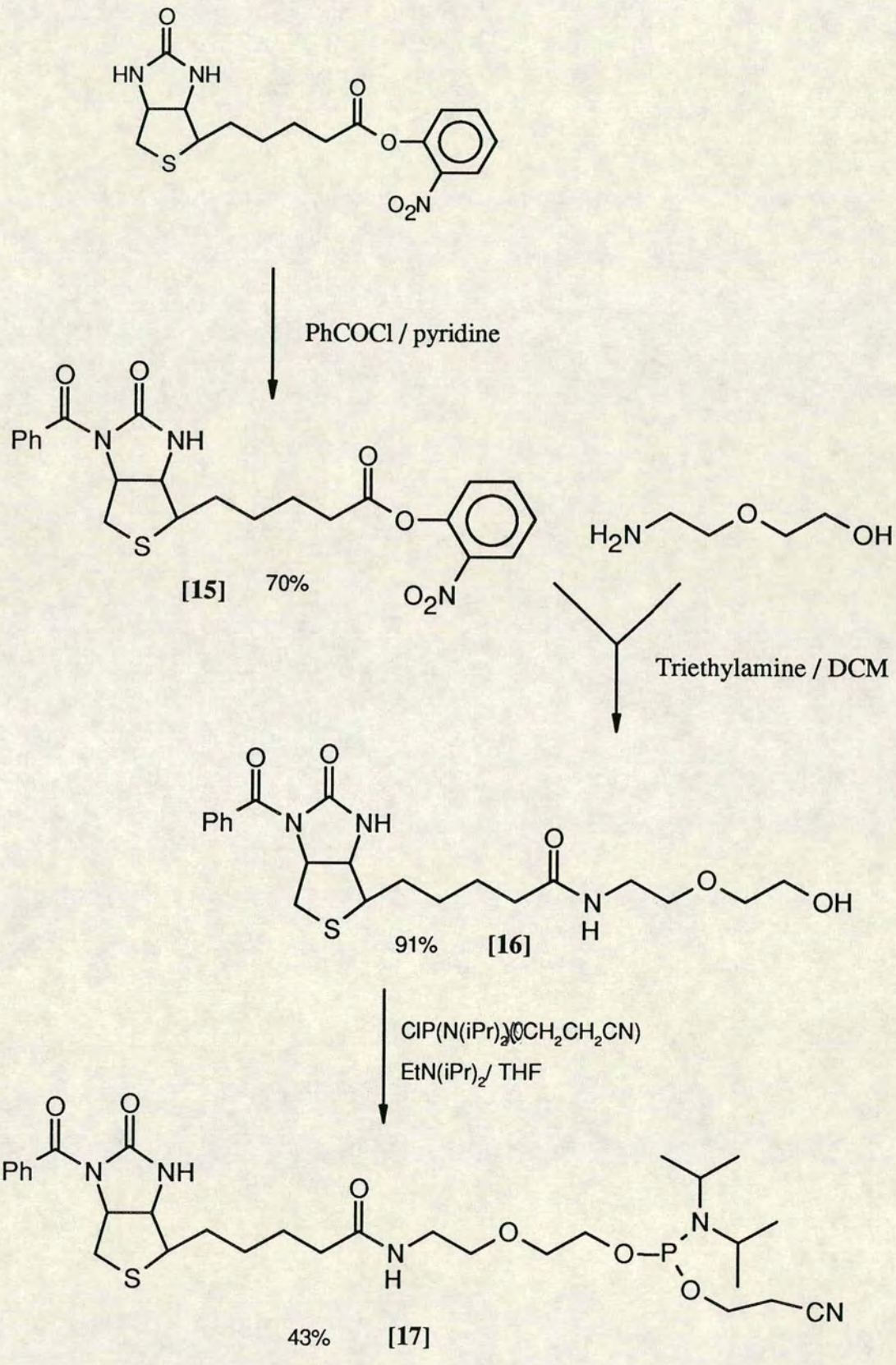
According to Misiura *et al.* [27] the attachment of multiple biotin residues does not give rise to an increase in sensitivity when a streptavidin-based detection system is used (Section 3.2.2.). Pieles *et al.* [93] demonstrated the necessity of protection of $N^{1'}$ of the biotin ring to prevent side-reactions (Section 3.2.2.). Based on these observations it was decided to attempt the synthesis of single addition protected biotin phosphoramidites.

9.1. *Chemical Synthesis of a 3-oxa-pentyl spaced biotin phosphoramidite.*[17]

A base-labile acyl protecting group for $N^{1'}$ of biotin was considered to be the most appropriate approach for the synthesis of a biotin phosphoramidite. The 4-*tert*-butyl benzoyl protecting group had been successfully used by Pieles *et al.* [93] for the protection of their multiple addition biotin phosphoramidite (*Figure 13*), although a rather lengthy synthetic route was chosen. It was decided to synthesize the $N^{1'}$-benzoyl derivative of a biotin active ester. This would allow the introduction of a protected biotin unit onto any amino function in the presence of unprotected hydroxyl functions.

Biotin-2-nitrophenyl ester (*Scheme 5*) was synthesized in high yield by the procedure of Pieles *et al* [93] and was reacted with benzoyl chloride in anhydrous pyridine to give $N^{1'}$-benzoylbiotin-2-nitrophenyl ester **[15]** in 70% yield. The product was readily identified on t.l.c., giving a yellow colour on treatment with conc. aqueous ammonia then a pink colour on heating with *p* -dimethylamino-cinnamaldehyde.[108,109]

As a first attempt at the synthesis of a benzoyl biotin phosphoramidite it was decided to use inexpensive 1-amino-3-oxa-pentan-1-ol as a spacer group. Thus $N^{1'}$-benzoylbiotin-2-nitrophenyl ester **[15]** was reacted with 1-amino-3-oxa-pentan-1-ol in the presence of triethylamine to give the benzoyl biotin alcohol **[16]** in excellent yield. Phosphitylation of **[16]** using 2-cyanoethyl N,N-diisopropyl phosphoramidochloridite in the presence of N,N-diisopropylethylamine gave the short chain biotin phosphoramidite **[17]** as a foam. This compound was found to be rather too polar for easy handling and purification and ^{31}P-NMR indicated that this phosphoramidite was contaminated by *ca.* 10% phosphonate impurities due to hydrolysis


PhCOCl / pyridine
[15] 70%
Triethylamine / DCM
91% [16]
ClP(N(iPr)2)(OCH2CH2CN)
EtN(iPr)2/ THF
43% [17]

Scheme 5

during aqueous work-up. This did not appear to interfere with the coupling of **[17]**.

9.2. *Oligonucleotide Synthesis using Biotin Phosphoramidite* [17].

Phosphoramidite **[17]**, as a 0.1M solution in anhydrous acetonitrile, was used to synthesize oligonucleotides under standard DNA synthesis conditions. The coupling efficiency was >95% (estimated by HPLC). In order to ascertain if the benzoyl group was fully removed under normal base deprotection conditions a test sequence **[17]**pTpTpTpTpTpT was synthesized. HPLC analysis of aliquots removed at one hour intervals from a solution of this oligonucleotide in NH_4OH heated to 55°C, indicated that the benzoyl group was completely removed under the conditions required to cleave the oligonucleotide from the solid support (1.5 hours in NH_4OH at room temperature). *Figure 34a* shows an HPLC chromatogram of a crude 26mer oligonucleotide (A; Appendix 1).

Biotinylated PCR primers were synthesized using either **[17]** or by conventional reaction of an Aminolink 2 (*Figure 7a*) oligonucleotide with biotin-NHS ester (to give a hexamethylene-spaced biotin). The presence of the unprotected biotin ring was confirmed by the generation of a pink colour after applying an aqueous solution of the oligonucleotide to a tlc plate and spraying with *p*-dimethylamino-cinnamaldehyde.[108,109] It was found that due to the more polar spacer arm incorporated using **[17]** gave poorer resolution of the biotinylated oligonucleotide from failure sequences by reversed-phase HPLC than the aminolink-biotinylated oligonucleotides (Which have an hexamethylene spacer arm). The separation of the failure sequences from the biotinylated oligonucleotide labelled with **[17]** was dependent upon the sequence and length of the

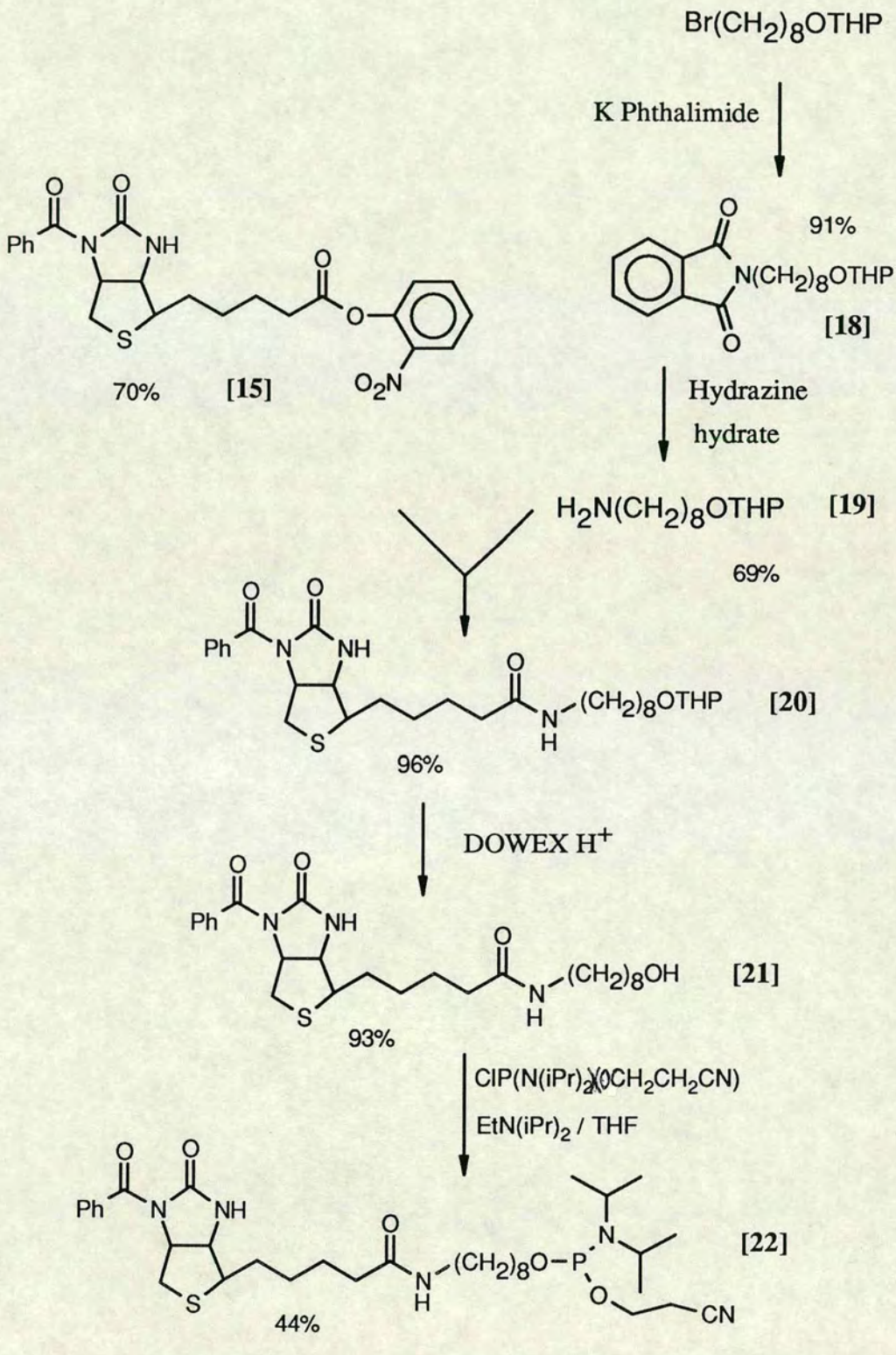
oligonucleotide. Clearly a longer, more lipophilic spacer arm would overcome this problem.

9.3. *Chemical Synthesis of an octamethylene spaced biotin phosphoramidite.* [22].

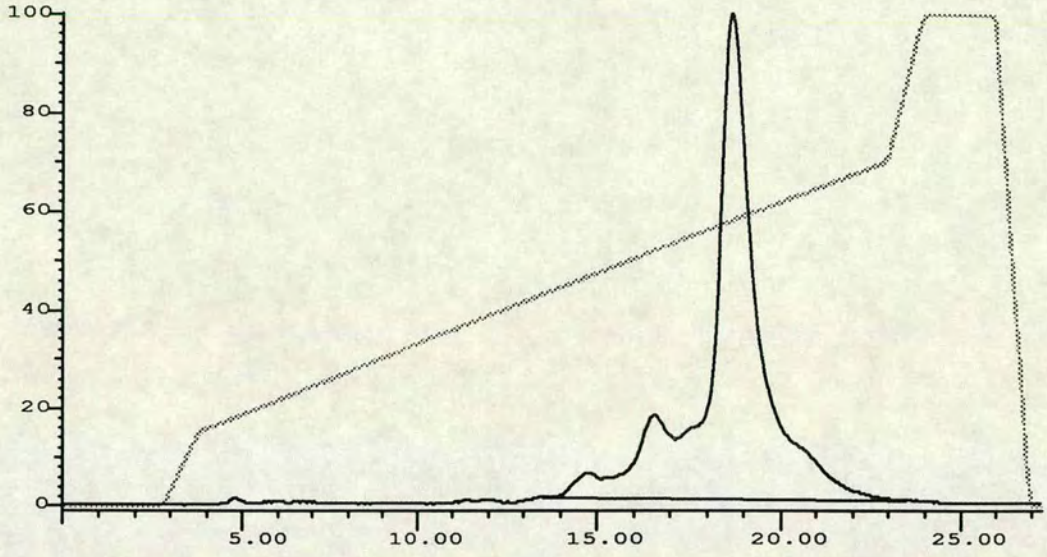
In order to improve upon the resolution of failure sequences from biotinylated oligonucleotide obtained from either the 3-oxa-pentyl spacer (introduced using **[17]**) or the hexamethylene spaced biotin (introduced using Aminolink-2, or the phosphoramidite of Pon[87]), it was decided to synthesize a benzoyl biotin phosphoramidite incorporating an octamethylene spacer arm.

THP protected aminooctanol was synthesized in two steps from commercially available 1-Bromo-8-tetrahydropyranyloxyoctane by the Gabriel method. (*Scheme 6*) The tetrahydropyranyl group, although not crucial to the synthetic strategy, was found to be convenient as this compound **[19]** was used in other applications (described in Chapter 2 of this thesis.) Thus 1-Bromo-8-tetrahydropyranyloxy-octane was reacted with potassium phthalimide in anhydrous dimethylformamide to give 1-phthalimido-8-tetrahydropyranyloxyoctane **[18]** in 91% yield. The phthaloyl group was removed by hydrazine hydrate in methanol to give, after distillation, 1-amino-8-tetrahydropyranyl-oxyoctane **[19]** in 69% yield.

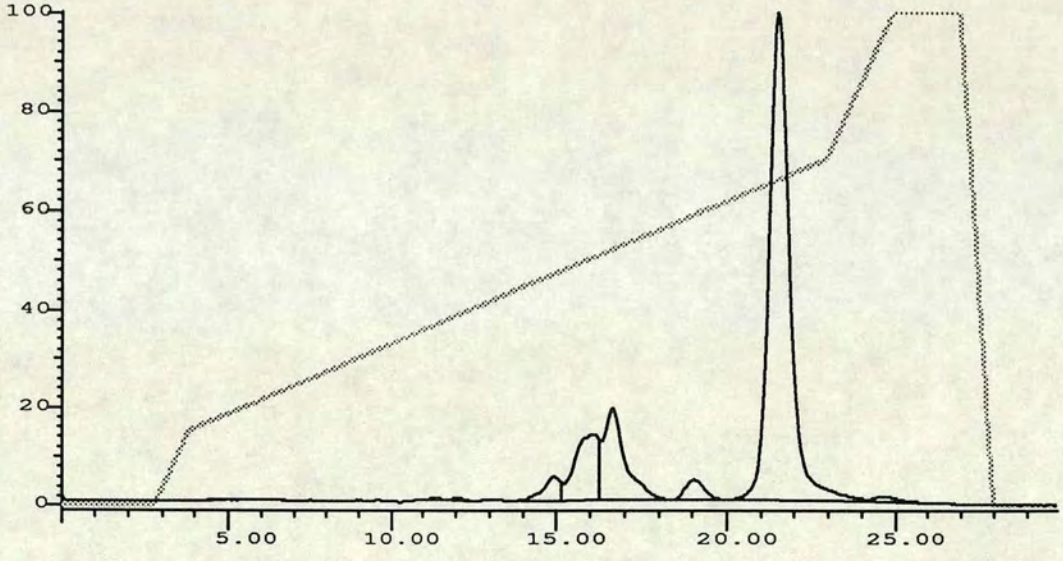
The benzoylated active ester **[15]** was then reacted with amine **[19]** in anhydrous tetrahydrofuran in the presence of triethylamine to give the amide **[20]** in 96% yield which, on treatment with DOWEX H^+ in methanol gave **[21]** (93%). Finally compound **[21]** was reacted with 2-cyanoethyl N,N-diisopropylphosphoramidochloridite in anhydrous tetrahydrofuran / N,N-diisopropylethylamine to give


Br(CH2)8OTHP
K Phthalimide
91%
N(CH2)8OTHP
[18]
Hydrazine
hydrate
H2N(CH2)8OTHP [19]
69%
Ph
NH
S
O2N
70%
[15]
(CH2)8OTHP
[20]
96%
DOWEX H+
(CH2)8OH
[21]
93%
ClP(N(iPr)2)(OCH2CH2CN)
EtN(iPr)2 / THF
(CH2)8O
P
CN
[22]
44%

Scheme 6



(a) HPLC chromatogram (preparative) of a crude 26mer oligonucleotide biotinylated using the 3-oxa-pentyl phosphoramidite **[17]**.



(b) HPLC chromatogram of a crude 24mer oligonucleotide biotinylated with the octamethylene phosphoramidite **[22]**.

Figure 34

8-(N-($N^{1'}$-benzoylbiotinyl)-1-aminooctyl)-2-cyanoethyl-N,N-diisopropylamino phosphoramidite **[22]** as a white powder in 44% yield after silica-gel chromatography. This phosphoramidite was sufficiently lipophilic to allow easy handling and purification.

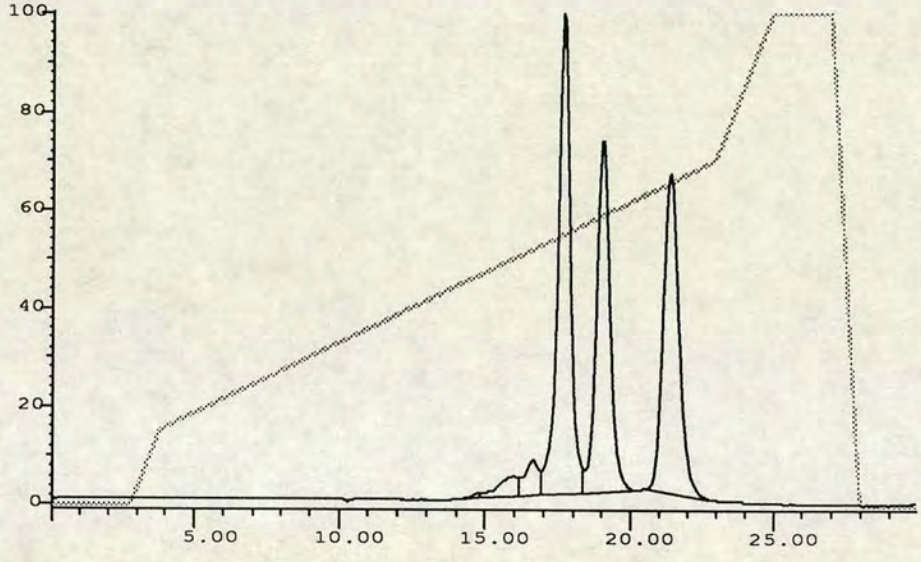
9.4. ***Oligonucleotide Synthesis using Biotin Phosphoramidite*** **[22].**

A 0.1M solution of **[22]** in anhydrous acetonitrile was used for standard 0.2 μmol and 1.0 μmol scale oligonucleotide synthesis on an Applied Biosystems 380B DNA synthesizer. The coupling efficiency of **[22]** was estimated to be >95% by HPLC. The benzoyl group was completely removed from $N^{1'}$ of the biotin moiety on the oligonucleotide after 1h at 20°C in conc. NH_4OH (HPLC monitoring) and the presence of the unprotected biotin ring on the oligonucleotide was confirmed by the generation of a pink colour after applying an aqueous solution of the oligonucleotide to a tlc plate and spraying with *p* -dimethylamino-cinnamaldehyde. Reversed-phase HPLC analysis of aliquots of **[22]**$p(Tp)_4T$ heated in conc. NH_4OH at 55°C for periods of 2 to 24h confirmed the aliphatic amide link to be stable to standard deprotection conditions. The octamethylene linker arm gave baseline resolution of product from failure sequences, which elute approximately 5 minutes earlier, for oligonucleotides up to *ca.* 35 bases long (*Figure 34b*). Such separation is not always possible with the hexamethylene spacer and never possible with the 3-oxa-pentyl linker. The relative retention times of a series of biotinylated 23mers (OSWEL 1, Appendix 1) with various linker arms were determined by HPLC. (*Figure 35*).

A PCR primer (27mer, OSWEL 2, Appendix 1) biotinylated with **[22]** was synthesized and supplied to a customer of the OSWEL DNA

Service. It was reported that this primer worked well in PCR amplification.

To confirm the presence of the biotin ring and spacer, 10 mg of biotinylated $(Tp)_4T$ were synthesized and purified and analysed by 600MHz ^1H NMR using COSY techniques to assign the spectrum. The assignment of the spectrum is given in the experimental section.



(a) (b) (c)

Figure 35 : HPLC chromatogram of biotinylated oligonucleotides (23mers) with different linkers (a) 3-oxa-pentyl; (b) hexamethylene; (c) octamethylene.

9.5. *Conclusions about Biotin Phosphoramidites* [17] *and* [22].

(i) The use of the benzoyl biotin active ester **[15]** is a convenient method of introducing a protected biotin unit onto an amine-containing backbone in one step and in high yield.

(ii) Both benzoyl biotin phosphoramidites were soluble in acetonitrile and coupled at >95%.

(iii) The $N^{1'}$-benzoyl protecting group is removed from the biotin moiety very rapidly by NH_4OH at room temperature.

(iv) The aliphatic amide link between the biotin and the spacer is stable to ammonia deprotection conditions.

(v) The 3-oxa-pentyl spacer arm introduced using **[17]** gives biotinylated oligonucleotides which are more lipophilic than failure sequences, however the differences in HPLC retention time are too short to allow convenient purification of oligonucleotides biotinylated using **[17]**.

(vi) The octamethylene spacer confers excellent physical properties to phosphoramidite **[22]** in terms of lipophilicity and solubility in acetonitrile.

(vii) Due to its increased lipophilicity the octamethylene spacer gives excellent separation of biotinylated oligonucleotides, making them easier to purify by reversed-phase HPLC than unmodified oligonucleotides under identical conditions.

(viii) PCR experiments carried out using primers biotinylated using **[22]** were successful.

(ix) The synthesis of multiple addition biotin phosphoramidites using the benzoyl biotin active ester **[15]** has yet to be attempted, but should be feasible.

(x) This work was presented as a poster at the San Diego Conference on Nucleic Acids 1991.

C. EXPERIMENTAL.

Solvents and Reagents.

Solvents were of laboratory grade, except those used for the extraction and chromatography of phosphoramidites, which were of HPLC grade. Pyrogen-free, reverse osmosis-purified water was used during oligonucleotide synthesis, analysis and purification.

Anhydrous solvents and reagents

Dichloromethane was distilled over CaH_2; tetrahydrofuran and diethyl ether were distilled over sodium/benzophenone; N,N-dimethylformamide was fractionally distilled under reduced pressure over 4A molecular sieve; pyridine was distilled over CaH_2; acetone was distilled over anhydrous $CaSO_4$ (Sikkon Fluka Universal Dessicant); hexane was dried over Na wire; triethylamine and N,N-diisopropylethylamine were dried over CaH_2; anhydrous N,N-dimethylacetamide was purchased from Aldrich; anhydrous acetonitrile was purchased from Applied Biosystems Inc. (ABI).

Other reagents.

2-cyanoethyl N,N-di-isopropyl phosphoramidochloridite was synthesized and purified by the procedure of Booth.[110] 1-Bromo-8-tetrahydropyranyl-oxyoctane and potassium phthalimide were supplied by Lancaster Synthesis. 4,4'-Dimethoxytrityl chloride was purchased from Fluka and recrystallised from hexane containing 1% acetyl chloride. *para* -Toluene sulphonyl chloride was purchased from Aldrich and recrystallised from petroleum ether/chloroform. All other chemicals were supplied by Aldrich or Fluka.

NMR

^{1}H-NMR spectra were recorded on a Brucker WP-80 spectrometer (80MHz) and a Brucker WP-200 spectrometer (200.130MHz); ^{13}C-NMR spectra were recorded on a Brucker WP-200 spectrometer (50.32MHz); and some ^{31}P-NMR spectra were recorded on a Brucker WP-200 spectrometer (81MHz). Other ^{31}P-NMR spectra were recorded on a Jeol FX90Q spectrometer (90MHz). Correlated Spectroscopy (COSY) experiments were carried out on a Varian Instruments VXR-600 (599.945 MHz). Nuclear Overhauser Effect experiments were carried out on a Bruker WH-360 (360MHz).

Mass Spectra.

Positive ion Fast Atom Bombardment (f.a.b.) mass spectra were recorded on a Kratos MS50 TC spectrometer using a thioglycerol or 3-nitrobenzyl alcohol (3-NOBA) matrix.

IR spectra.

IR spectra were recorded on a Perkin-Elmer 781 IR spectrophotometer using KBr plates and a polystyrene reference (1603 cm^{-1} and 1029cm^{-1}).

Melting Points.

Melting points were determined on a Koffler Hot Stage Microscope apparatus and are uncorrected.

Oligonucleotide synthesis.

Oligonucleotide synthesis was performed on an Applied Biosystems 380B DNA synthesizer. All DNA synthesis reagents and cyanoethyl-phosphoramidite monomers were supplied by ABI or Cruachem.

Measurement of phosphoramidite coupling efficiencies.

Coupling efficiencies of phosphoramidites were measured by comparison of the absorbance at 498nm of the dimethoxytrityl cations produced in the detritylation steps of successive synthesis cycles. The coupling efficiency was calculated as follows:

$[A_{498}(\text{cycle n+1})/A_{498}(\text{cycle n})]\text{x}100$

The absorbances were measured by dilution of the detritylation product fractions to 25ml with 0.1M Toluene-4-sulphonic acid in acetonitrile. Coupling efficiencies of phosphoramidites with more than one dimethoxytrityl group were calculated by division of the absorbance at 498nm by the number of dimethoxytrityl groups present. Coupling efficiencies of phosphoramidites (X) without a dimethoxytrityl group were estimated by comparison of the integrals of the HPLC peaks for a test sequence XT_5 and the failure sequence T_5.

Oligonucleotide analysis and purification.

Reversed-phase HPLC analysis and purification were carried out on a Gilson model 306 HPLC system using ABI Brownlee Aquapore Octyl reverse phase columns. Oligonucleotides >12 bases long were desalted on NAP-10 columns (Sephadex G25) (Pharmacia) following manufacturers instructions. Shorter oligonucleotides were desalted on a Sephadex G-10 column (35cm x 2cm) using a 1 ml min^{-1} flow rate.

Buffer systems:

Buffer A-0.1M NH_4OAc; Buffer B-0.1M NH_4OAc/X% acetonitrile, where 20<X<65%, depending upon the lipophilicity of the oligonucleotide. (Exact buffer systems are reported in each case.)

Gradients :

Gradient A.

Time/min	Flow/mlmin^{-1}	%Buffer B
0	3	0
3	3	0
4	3	15
23	3	70
25	3	100
28	3	100
29	3	0
30	3	0

Gradient B.

Time/min	Flow/mlmin^{-1}	%Buffer B
0	3	0
3	3	0
4	3	15
23	3	100
27	3	100
28	3	0
30	3	0

Gradient C.

Time/min	Flow/mlmin^{-1}	%Buffer B
0	3	0
3	3	0
4	3	15
19	3	100
28	3	100
29	3	0
35	3	0

Detritylation of "trityl-on" oligonucleotides.

After HPLC purification "trityl-on" oligonucleotides were evaporated to dryness and taken-up in 3% aqueous acetic acid (4ml). After 1h at 20°C the solutions were evaporated to dryness then desalted on Sephadex G-25. Removal of the dimethoxytrityl group was confirmed by HPLC.

Chromatography.

Flash chromatography was carried out using silica gel 60 (Fluka). Thin layer chromatography (tlc) was carried out on aluminium sheets, silica 60 F_{254}, 0.2mm layer (Merck) using the following solvent systems:

(A) Toluene-ethyl acetate (50:50, v/v)

(B) Toluene-ethyl acetate (80:20, v/v)

(C) Dichloromethane-methanol (90:10, v/v)

(D) Dichloromethane-methanol (99:1, v/v)

(E) Dichloromethane-isopropanol (95:5, v/v)

(F) Dichloromethane-methanol (80:20, v/v)

(G) n-Butanol-acetic acid-water (60:20:20, v/v/v)

(H) Dichloromethane-ethyl acetate (50:50, v/v)

(I) Dichloromethane-methanol (95:5, v/v)

(J) Hexane-ethyl acetate (50:50, v/v)

(K) Ethyl acetate-methanol-NH_4OH (5:1:1, v/v/v)

(L) Ethyl acetate-acetonitrile (50:50, v/v)

(M) Dichloromethane-acetonitrile (50:50, v/v)

(N) Hexane-ethyl acetate (80:20, v/v).

Products were visualised on tlc using the following techniques:

(i) UV absorbtion at 264nm where appropriate.

(ii) Spraying with a 10% solution of phosphomolybdic acid in ethanol. Gives a dark blue colour for oxidisable compounds.

(iii) Spraying with a 0.2% solution of *p* -dimethylaminocinnamaldehyde and 2% sulphuric acid in ethanol.[108,109] Gives a pink colour for biotin containing compounds and biotin containing oligonucleotides. Compounds with a $N^{1'}$-protecting group on biotin require prolonged heating to develop the colour.

(iv) Spraying with a solution of *para* - methoxybenzaldehyde:acetic acid:H_2SO_4: ethanol (5:1:1:100, v/v/v/v). Compounds with a protected or unprotected 1,2-diol function give a dark blue colour on heating.

(v) Spraying with a 1% solution of ninhydrin in ethanol. Amines give a strong purple/brown colour on heating. Amides and phthalimides give a brown colour on strong heating.

(vi) Dinitrophenyl-containing compounds can be seen directly due to their bright yellow colour.

3,6-diazaoctane-1,8-diol derivatives

3,6-Bis(2,4-dinitrophenyl)-3,6-diazaoctane-1,8-diol [1].

N,N'-Bis(2-hydroxyethyl)-ethylene diamine (1eq;1.5g;10mmol) was dissolved in methanol (30ml) and to this solution was added triethylamine (2.5eq; 2.53g; 3.48ml; 25mmol) and 2,4-dinitrofluorobenzene (2eq; 3.72g; 20mmol). After 2h the product appeared as an orange precipitate. After 3.5h the flask was cooled to 0°C in an ice bath and the precipitate was filtered-off and washed with methanol (30ml) and diethyl ether (3x30ml). After drying *in vacuo* the product was recrystallized from methanol to give the *title compound* **[1]** as dark orange crystals (3.89g; 81%). m.p. 149-150°C; (Found C, 44.8; H, 4.18; N, 17.4. $C_{18}H_{20}N_6O_{10}$ requires C,45.0; H, 4.16; N, 17.5%); R_f 0.38 (Solvent C). ν_{max}(nujol)/cm^{-1} 3490 (m, OH), 2850 (s, aliphatic), 1600 (s, aromatic), 1580 (m), 1525 (s), 1495 (s), 1470 (s), 1420 (w), 1395 (w), 1360 (m), 1330 (s), 1270 (s), 1230 (w), 1205 (m), 1165 (w), 1145 (m), 1125 (w), 1080 (w), 1070 (w), 1045 (w), 995 (w), 930 (w), 915 (w), 900 (w), 850 (w), 830 (w), 770 (w), 745 (m), 725 (w), 655 (w), 630 (w). δ_H (80.13MHz, $CDCl_3$) 3.0-4.0 (12H, m, CH_2), 4.7 (2H, t, OH, J_{OH-CH2}=5Hz), 7.33 (2H, d, DNP 6-H,J_{5-6} =9.5Hz), 8.13 (2H, dd, DNP 5-H, $J_{5-H-6-H}$=9.5Hz, J_{3-H-5H} =2.8Hz), 8.47 (2H, d, DNP 3-H, $J_{3-H-5-H}$=2.8Hz). f.a.b. ms, *m/z* 481 $(M+H)^+$.

8-(4,4'-Dimethoxytrityloxy)-3,6-bis(2,4-dinitrophenyl)-3,6-diazaoctan-1-ol [2]

3,6-Bis(2,4-dinitrophenyl)-3,6-diazaoctane-1,8-diol **[1]** (1eq;3.30g; 6.88mmol) was dried by evaporation of anhydrous pyridine (3 x 30ml) and dissolved in anhydrous pyridine (60ml). To this solution was added dropwise over 30 min, a solution of 4,4'-dimethoxytrityl chloride (1.3eq;

3.03g; 8.94mmol) in anhydrous pyridine (100ml). After 2hr a second aliquot of 4,4'-dimethoxytrityl chloride (0.2eq; 0.47g; 1.38mmol) in anhydrous pyridine (20ml) was added dropwise over 20 min and after a further 30 min the reaction was quenched with methanol (20ml) and the solvent was evaporated *in vacuo*. The residue was dissolved in dichloromethane (250ml) and washed with saturated aqueous $NaHCO_3$ (50ml), and water (3 x 50ml). The organic phase was dried (Na_2SO_4), filtered and the solvent removed *in vacuo*. The residue was dissolved in the minimum volume of toluene-ethyl acetate (1:1, v/v)+1% triethylamine and applied to a silica gel chromatography column, eluting with the same solvent system, to give the *title compound* **[2]** as an orange foam (1.68g; 31%). R_f 0.29 (solvent D); R_f 0.58 (solvent E); R_f 0.35 (solvent A). ν_{max}(nujol)/cm^{-1} 3560 (w, OH), 1605 (s, aromatic), 1590 (s), 1500 (s), 1330 (s), 1245 (s), 1170 (m), 1145 (m), 1125 (w), 1060 (w), 1030 (m), 990 (w), 930 (w), 910 (w), 830 (m), 790 (w), 745 (m), 720 (m). δ_H(80.13MHz; $CDCl_3$) 2.0-2.25 (1H, bs, OH), 3.25-3.72 (12H, m, CH_2), 3.73 (6H, s, OCH_3), 6.65-7.23 (15H, m, DMT, DNP 6-H), 8.06 (1H, dd, DNP 5-H, $J_{5\text{-}H\text{-}6\text{-}H}$=9.3Hz, $J_{3\text{-}H\text{-}5\text{-}H}$=2.7Hz), 8.13 (1H, dd, DNP 5-H, $J_{5\text{-}H\text{-}6\text{-}H}$=9.3Hz, $J_{3\text{-}H\text{-}5\text{-}H}$=2.3Hz), 8.52 (d, 1H, DNP 3-H, $J_{3\text{-}H\text{-}5\text{-}H}$=2.7Hz), 8.55 (1H, d, DNP 3-H, $J_{3\text{-}H\text{-}5\text{-}H}$=2.7Hz). f.a.b. ms, *m/z* 783.26259. [$(M+H)^+$, $C_{39}H_{39}N_6O_{12}$ requires 783.26257].

2-Cyanoethyl-[8-(4,4'-dimethoxytrityloxy)-3,6-bis(2,4-dinitrophenyl)-3,6-diazaoctyl] N,N-di-isopropylphosphoramidite [3]

8-(4,4'-Dimethoxytrityloxy)-3,6-bis(2,4-dinitrophenyl)-3,6-diazaoctan-1-ol **[2]** (1eq;0.643g; 0.822mmol) was dried by evaporation of anhydrous tetrahydrofuran (3 x 20ml) and dissolved in anhydrous tetrahydrofuran (30ml). To this solution was added N,N-diisopropylethylamine(4eq;

3.29mmol; 0.425g; 0.573ml) and 2-cyanoethyl N,N-di-isopropyl-phosphoramidochloridite (1.5 eq; 1.23mmol; 0.291g; 0.276ml). The solution was stirred at 20°C and the reaction was complete by tlc after 15 min. The reaction was diluted with ethyl acetate (30ml) and the resulting solution was washed with brine (4 x 100ml). The organic layer was dried (Na_2SO_4), filtered and the solvent was removed *in vacuo*. The residue was dissolved in the minimum volume of toluene-ethyl acetate (80:20, v/v)+1% triethylamine, and applied to a silica gel chromatography column, eluting with the same solvent system, to give **[3]** as an orange foam. The foam was dissolved in the minimum volume of anhydrous dichloromethane and the solution was added dropwise to rapidly stirred anhydrous hexane at -78°C. The precipitated product was collected by filtration under a stream of argon, to give the *title compound* **[3]** as an orange powder (0,678g; 84%). R_f 0.50 (solvent B). δ_P (81.020 MHz; $CDCl_3$) 149.12 (s). f.a.b. ms *m/z* 983.37044 [$(M+H)^+$,$C_{48}H_{56}N_8O_{13}P$ requires 983.37042].

6-aminohexan-1-ol derivatives.

6-(2,4-Dinitrophenylamino) hexan-1-ol [4].

6-Aminohexan-1-ol,(1eq; 0.8g; 6.8mmol) was dissolved in methanol (20ml). To this solution was added 2,4-dinitrofluorobenzene (1eq; 1.3g). After 12hr the solvent was evaporated *in vacuo* and the resulting oil was washed with diethyl ether. Crude yield of **[4]** was 70% (1.3g). Recrystallisation from diethyl ether/ methanol gave the *title compound* **[4]** as waxy yellow crystals(1.04g; 54%). m.p. 75°C; (Found C, 50.65; H, 5.83; N, 14.78. $C_{12}H_{17}N_3O_5$ requires C, 50.88; H, 6.01; N, 14.84%); R_f 0.52 (solvent C); ν_{max}(nujol)/cm^{-1} 3530 (m, OH), 3380 (m, NH), 3110 (w, NH), 2850 (s, aliphatic), 1605 (s, aromatic), 1520 (s), 1490 (s), 1460

(s), 1415 (m), 1375 (s), 1335 (s), 1305 (s), 1265 (s), 1240 (s), 1225 (s), 1165 (m), 1140 (s), 1125 (m), 1100 (m), 1070 (s), 1060 (m), 1035 (m), 980 (m), 935 (m), 920 (s), 870 (w), 860 (w), 835 (s), 765 (m), 745 (s), 700 (s), 645 (w), 610 (s). δ_H(200.130MHz; $CDCl_3$) 1.1-2.0 (9H, m, CH_2, OH), 3.25-3.5 (2H, m, C^1H_2), 3.64 (2H, t, C^6H_2, J=6.2Hz), 6.9 (1H, d, Ar H-6, J_{ortho}=9.56Hz), 8.25 (1H, ddd, Ar H-5, J_{ortho}=9.53Hz, J_{meta}=2.69Hz, J_{para}=0.75Hz), 8.5 (1H,br s, NH), 9.1 (1H, d, Ar H-3, J_{meta}=2.66Hz); δ_C(50.320MHz, $CDCl_3$) 25.24 (CH_2),26.55 (CH_2), 28.48 (CH_2), 32.25 (CH_2), 43.35 (CH_2), 62.42 (CH_2), 113.82 (CH), 124.15 (CH), 130.17 (CH+C), 135.68 (C), 148.23 (C); f.a.b. ms *m/z* 284.12462 [$(M+H)^+$, $C_{12}H_{18}N_3O_5$ requires 284.12463].

1-(2-Cyanoethyl-6-(2,4-dinitrophenylamino)hexyl)-N,N-diisopropylphosphoramidite [5].

6-(2,4-Dinitrophenylamino) hexan-1-ol **[4]** (1eq;0.14g; 0.495mmol) was dissolved in anhydrous tetrahydrofuran (5ml) and to this solution was added anhydrous diisopropylethylamine (1eq;0.1ml) and 2-cyanoethyl N,N-di-isopropyl phosphoramidochloridite (1.2eq;0.14g; 0.132ml). After 1h the reaction was diluted with ethyl acetate (20ml) and washed with $NaHCO_3$ (2x5ml) and brine (2x5ml). The organic phase was dried (Na_2SO_4), filtered and evaporated *in vacuo*. The resulting oil was dissolved in the minimum volume of dichloromethane: ethyl acetate, 1:1, and applied to a flash chromatography column, eluting with the same solvent system, to give **[5]** as an oil. This was dissolved in dichloromethane and precipitated from anhydrous hexane to give the *title compound* **[5]** as an red-orange oil (0.1g; 80%). R_f 0.8 (solvent F); δ_P(81MHz; $CDCl_3$)147.54 (s); f.a.b. ms *m/z* 484.23247 [$(M+H)^+$, $C_{21}H_{35}N_5O_6P$ requires 484.23248].

HPLC analysis and Purification of Oligonucleotides DNP-labelled using [3] and [5].

No. of DNP groups	Phosphoramidite used.	Gradient used	%MeCN in Buffer B.
1	**[5]**	A	20
2	**[3]**	B	20
4	**[3]**	B	40
6	**[3]**	B	50
10	**[3]**	B	65

Multiple Addition DNP Phosphoramidite based on a 1,2,6-Trihydroxyhexane Backbone.

5,6-Isopropylidenedioxyhexan-1-ol [6].

1,2,6-Trihydroxyhexane (37g; 0.27mol) was dissolved in anhydrous acetone (250ml) and to this solution was added anhydrous sodium sulphate (100g) and conc. aqueous HCl (5ml). After stirring overnight at 20°C, lead (II) carbonate (basic form) (100g) and Na_2CO_3 (1g) were added and the reaction was left for a further 48h. Solids were removed by filtration and the solvent was evaporated *in vacuo* to give a pale yellow liquid. Na_2CO_3 (1g) was added to the liquid to maintain basic conditions and the residue was purified by kugelrohr distillation to give the *title compound* **[6]** as a clear liquid (39.92g, 85%). b.p. 120°C (at 0.05mbar). ν_{max}(film)/cm^{-1} 3410 (s, OH), 2985 (s, CH), 2930 (s, CH), 2860 (s, CH), 1455 (m), 1380 (s), 1370 (s), 1250 (s), 1215 (s), 1155 (s), 1050 (s), 915 (w), 855 (m), 815 (w), 790 (w), 735 (w). R_f 0.46 (solvent C); δ_H(200.130MHz; $CDCl_3$) 1.24 (3H,s,CH_3), 1.29 (3H,s,CH_3), 1.24-1.60 (6H,m,CH_2), 2.91 (1H,brs,OH), 3.36-3.52 (3H,m,CH,CH_2), 3.89-4.01

(2H,m,CH_2); δ_C(50.32MHz; $CDCl_3$) 21.73(CH_2), 25.39 (CH_3), 26.59 (CH_3), 32.21 (CH_2), 32.93 (CH_2), 61.89 (CH_2), 69.06 (CH_2), 75.71 (CH), 108.41 (C). f.a.b. ms *m/z* 175.133411 [$(M+H)^+$, $C_9H_{19}O_3$ requires 175.13341].

5,6-Isopropylidenedioxyhex-1-yl *p*-toluenesulphonate [7].

To a solution of compound **[6]** (1eq; 2.3g; 0.014mol) in anhydrous pyridine (50ml) cooled to 0°C, was added 4-toluene sulphonyl chloride (1eq; 0.014mol; 2.74g). After 2h at 20°C the solution was evaporated to an oil which was coevaporated with toluene three times. The residue was taken-up in dichloromethane and washed with water (50ml) and sat. aq. KCl (3 x 50ml). The organic phase was dried (Na_2SO_4), filtered and the solvent removed by evaporation *in vacuo*. The product was purified by wet-flash silica gel chromatography, eluting with 10% ethyl acetate in toluene, to give the *title compound* **[7]** as a pale yellow clear oil (2.874g; 61%). R_f 0.40 (solvent B); ν_{max}(film)/cm^{-1} 2980 (s, CH), 2930 (s, CH), 2870 (s, CH), 1600 (m, aromatic), 1495 (w), 1455 (m), 1360 (s, sulphonyl), 1308 (w), 1295 (w), 1250 (m), 1210 (s), 1190 (s), 1175 (s, sulphonyl), 1095 (s), 1055 (s), 960 (s), 930 (s), 835 (m), 815 (m), 775 (m), 730 (m), 705 (w), 690 (w), 665 (s). δ_H(200.130MHz; $CDCl_3$) 1.24 (3H,s,CH_3), 1.29 (3H,s,CH_3), 1.24-1.63 (6H,m,CH_2), 2.36 (3H,s, Ar-CH_3), 3.36-3.41 (1H,m,CH), 3.87-3.97 (4H,m,CH_2), 7.26 (2H,d, Ar 3-H, Ar 5-H, J_{ortho}=7.84Hz), 7.69 (2H,m,Ar 2-H, Ar 6-H); δ_C(50.32MHz; $CDCl_3$) 21.26 (CH_3), 21.41(CH_2), 25.34 (CH_3), 26.58 (CH_3), 28.40 (CH_2), 32.48 (CH_2), 68.93 (CH_2), 69.99 (CH_2), 75.31 (CH), 108.32 (C), 127.49 (2xAr CH), 129.54 (2xAr CH), 132.72 (C), 144.44 (C); f.a.b. ms *m/z* 329.14224 [$(M+H)^+$, $C_{16}H_{25}O_5S$ requires 329.14226].

N-Phthaloyl-5,6-isopropylidenedioxyhexylamine [8].

To a solution of compound [7] (1eq; 3.446g; 0.0105mol) in anhydrous N,N-dimethylformamide (15ml) was added potassium phthalimide (1.1eq; 0.0116mol; 2.14g). The reaction was stirred for 16h at 20°C then heated to 60°C for 2h after which time the reaction was found to be complete by tlc. The solvent was removed by evaporation to dryness and the residue was coevaporated once with toluene, dissolved in dichloromethane and washed with water (3 x 50ml). The organic phase was dried ($MgSO_4$), filtered and the solvent removed by evaporation *in vacuo* to give a pale brown solid. The product was purified by wet-flash silica gel chromatography, eluting with 10% ethyl acetate in toluene, to give the *title compound* [8] as a waxy white solid (3.037g; 96%). R_f 0.37 (solvent B). ν_{max}(nujol)/cm^{-1} 1720 (s, cyclic imide CO), 1700 (s, cyclic imide CO), 1400 (m), 1335(m), 1285(w), 1245(w), 1230(m), 1205(w), 1160(m), 1085(m), 1055(m), 1040(s), 985(w), 915(w), 890(w), 855(m), 790(w), 720(s). δ_H(200.130MHz; $CDCl_3$) 1.24 (3H,s,CH_3), 1.29 (3H,s,CH_3), 1.30-1.71 (6H,m,CH_2), 3.37-3.46 (1H,m,CH), 3.60 (2H, t, N-CH_2, ^3J=7.13Hz), 3.89-4.01 (2H,m,CH_2), 7.60-7.78 (4H,m,Ar CH); δ_C(50.32MHz; $CDCl_3$) 22.78(CH_2), 25.40 (CH_3), 26.61(CH_3), 28.25 (CH_2), 32.80 (CH_2), 37.42 (CH_2), 69.05 (CH_2), 75.47 (CH), 108.34 (C), 122.83 (2xAr CH), 131.79 (2xAr C), 133.56 (2xAr CH), 168.01 (2xCO); f.a.b. ms *m/z* 304.15488[$(M+H)^+$, $C_{17}H_{22}NO_4$ requires 304.15487].

5, 6-Isopropylidenedioxyhexylamine [9].

To a solution of compound [8] (1eq; 2.501g; 8.25mmol) in methanol (10ml) was added hydrazine hydrate (1.1eq; 9.08mmol; 0.454g; 0.44ml). The mixture was stirred for 16h at 20°C, during which time a white precipitate formed. The solvent was removed by evaporation *in vacuo*,

and the residue was dissolved in diethyl ether and washed with 2M NaOH. The aqueous phase was extracted three times with diethyl ether and the combined organic phase was dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a white solid which was purified by kugelrohr distillation to give the *title compound* **[9]** as a clear liquid (0.720g; 51%). b.p. 100°C (at 0.01mbar). R_f 0.36 (solvent K); ν_{max}(film)/cm^{-1} 3360 (s), 2985 (s), 2930 (s), 2860 (s), 1570 (s), 1485 (s), 1380 (s), 1370 (s), 1320 (s), 1245 (s), 1210 (s), 1160 (s), 1055 (s), 850 (s), 820 (m), 790 (w). δ_H(200.130MHz; $CDCl_3$) 1.27 (3H,s,CH_3), 1.32 (3H,s,CH_3), 1.17-1.63 (6H,m,CH_2), 1.49 (2H,s,NH_2), 2.62 (2H, t, N-CH_2, ^3J=6.6Hz), 3.32-3.46 (1H,m,CH), , 3.92-4.03 (2H,m,CH_2); δ_C(50.32MHz; $CDCl_3$) 22.88(CH_2), 25.47 (CH_3), 26.69(CH_3), 33.18 (CH_2), 33.39 (CH_2), 41.73 (CH_2), 69.19 (CH_2), 75.74 (CH), 108.37 (C); f.a.b. ms *m/z* 174.149392[$(M+H)^+$, $C_9H_{20}NO_2$ requires 174.14939].

1-(2,4-Dinitrophenylamino)-5, 6-isopropylidenedioxyhexane [10].

To a solution of compound **[9]** (1eq; 0.5g; 2.89mmol) in methanol (10ml) was added triethylamine (4eq; 0.012mol; 1.169g; 1.61ml) and a solution of 2,4-dinitrofluorobenzene (1eq; 2.89mmol; 0.538g) in methanol (5ml). The mixture was stirred at 20°C for 16h. Solvents were removed by evaporation *in vacuo*, and the residue was coevaporated 3 times with toluene. The residue was purified by wet-flash silica gel chromatography, eluting with a gradient of 5-10% ethyl acetate in toluene, to give the *title compound* **[10]** as an orange oil (1.101g; 100%). ν_{max}(film)/cm^{-1} 3360(m, NH), 3100(w, NH), 2980(m, CH), 2930(m, CH), 2840(m, CH), 1615(s, NO_2), 1590(s, aromatic), 1520(s), 1500(m), 1420(m), 1365(m), 1335(s, NO_2), 1310(s), 1240(m), 1150(m), 1130(m), 1055(m), 920(w), 850(w), 830(w), 820(w), 740(m), 710(w). R_f 0.33 (solvent B);

δ_H(200.130MHz; $CDCl_3$) 1.32 (3H,s,CH_3), 1.38 (3H,s,CH_3), 1.21-1.88 (6H,m,CH_2), 3.37-3.55 (3H,m,CH,CH_2), 3.98-4.12 (2H,m,CH_2) 6.89 (1H,d, DNP 6-H,$J_{5\text{-}H\text{-}6H}$=9.5Hz), 8.22 (1H,dd, DNP 5-H, $J_{5\text{-}H\text{-}6\text{-}H}$=9.5Hz, $J_{3\text{-}H\text{-}5H}$=2.6Hz), 8.54 (1H, bt, NH), 9.07 (1H,d, DNP 3-H, $J_{3\text{-}H\text{-}5\text{-}H}$=2.6Hz). δ_C(50.32MHz; $CDCl_3$) 23.11(CH_2), 25.45 (CH_3), 26.75(CH_3), 28.47 (CH_2), 32.88 (CH_2), 43.25 (CH_2), 69.11 (CH_2), 75.44 (CH), 108.69 (C), 113.72 (DNP-CH), 124.12 (DNP-CH), 130.14 (DNP-CH+C), 135.69 (DNP-C), 148.14 (DNP-C); f.a.b. ms *m/z* 340.15088 [$(M+H)^+$, $C_{15}H_{22}N_3O_6$ requires 340.15085].

1-(4, 4'-Dimethoxytrityloxy)-6-(2, 4-dinitrophenylamino) hexan-2-ol [11].

To a solution of compound **[10]** (1eq; 1.00g; 2.95mmol) in tetrahydrofuran (10ml) was added water (4ml) and conc. aqueous HCl (1ml). The reaction, to give the diol, was complete after 45min. R_f (diol) 0.39 (solvent C). The solvents were removed by evaporation *in vacuo* and the residue was coevaporated with anhydrous pyridine (4 x 10ml), then dissolved in anhydrous pyridine (20ml). To this solution was added 4,4'-dimethoxytrityl chloride (1.1eq; 3.25mmol; 1.1g). The mixture was stirred at 20°C for 16h then quenched with methanol (1ml). The solvent was removed by evaporated *in vacuo*. The residue was coevaporated with toluene (3 x 10ml), dissolved in dichloromethane and washed with sat. $NaHCO_3$(50ml) then sat. KCl (3 x 50ml). The organic phase was dried ($MgSO_4$), filtered and the solvent was removed by evaporation *in vacuo* . The residue was dissolved in hexane:ethyl acetate/ 1:1 and applied to a wet-flash silica gel chromatography column which had been equilibrated with hexane containing 20% ethyl acetate and 1% triethylamine. The column was then eluted with a gradient of 20-50% ethyl acetate in

hexane, to give the *title compound* **[11]** as a yellow foam (1.274g; 72%). R_f 0.26 (solvent J), R_f 0.71 (solvent H); ν_{max}(nujol)/cm^{-1} 3560 (w, OH), 3360(m, NH), 3160(w, NH), 2720(m), 1720 (w), 1620 (m, NO_2), 1590(m, aromatic), 1335(s, NO_2), 1300(s), 1245(s), 1170(m) 1150(m), 1115(w), 1075(w), 1030 (m), 970 (w), 920(w), 830(m), 790(w), 740(m), 720(s). δ_H(200.130MHz; $CDCl_3$) 1.45-1.74 (6H,m,CH_2), 2.44 (1H, bs,OH), 2.99-3.21 (2H,m,CH_2), 3.37 (2H, dt, CH_2-N, $J_{CH2-CH2}$=6.95Hz, J_{CH2-NH}=5.3Hz) , 3.71-3.78 (7H,m,CH+OCH_3), 6.79-6.89 (4H, m, DMT-CH), 7.14-7.45 (10H,m, DNP 6-H, DMT-CH), 8.24 (1H,dd, DNP 5-H, $J_{5-H-6-H}$=9.3Hz, J_{3-H-5H}=2.5Hz), 8.53 (1H, bt, NH), 9.12 (1H,d, DNP 3-H, $J_{3-H-5-H}$=2.7Hz). δ_C(50.32MHz; $CDCl_3$) 22.83 (CH_2), 28.51 (CH_2), 32.54 (CH_2), 43.34 (CH_2), 55.08 (2xDMT-CH_3), 67.34 (CH_2), 70.48 (CH), 86.04 (DMT-C), 113.02 (4xDMT-CH), 113.73 (DNP-CH), 124.21 (DNP-CH), 126.74 (DMT-CH), 127.73 (2xDMT-CH), 127.96 (2xDMT-CH), 129.18 (4xDMT-CH), 130.18 (DNP-CH+C), 135.79 (DNP-C, 2xDMT-C), 144.61 (DMT-C), 148.14 (DNP-C), 158.40 (2xDMT-C). f.a.b. ms *m/z* 601.24243 [M^+, $C_{33}H_{35}N_3O_8$ requires 601.24240].

2-Cyanoethyl [1-(4, 4'-dimethoxytrityloxy)-6-(2, 4-dinitrophenylamino) hex-2-yl] N,N-diisopropylamino phosphoramidite [12].

To a solution of compound **[11]** (1eq; 0.718g; 1.19mmol) in anhydrous tetrahydrofuran (10ml) was added N,N-diisopropylethylamine (4eq; 4.78mmol; 0.62g; 0.83ml) and 2-cyanoethyl N,N-di-isopropyl-phosphoramidochloridite (1eq; 1.19mmol; 0.283g; 0.266ml). After 1h the reaction was diluted with ethyl acetate (100ml) and the resulting solution was washed with sat. aqueous KCl(4 x 50ml). The organic phase was

dried ($MgSO_4$), filtered and solvents removed by evaporation *in vacuo* . A silica gel column was pre-equilibrated with hexane containing 40% ethyl acetate and 1% triethylamine and the residue was then purified on this column, eluting with 40% ethyl acetate in hexane, to give the *title compound* **[12]** as an orange gum (0.851g; 89%). R_f 0.54 (solvent J), R_f 0.60 (solvent B); δ_P(80MHz; CH_3CN solvent; $(CD_3)_2CO$ int) 147.57(s), 147.74 (s). f.a.b. ms *m/z* 803.36588 [$(M+2H)^+$, $C_{42}H_{54}N_5O_9P$ requires 803.36589].

Oligonucleotide Synthesis using DNP Phosphoramidite [12].

A 0.15M solution of **[12]** in anhydrous acetonitrile was used for oligonucleotide synthesis. Standard synthesis cycles for 0.2 µmol and 1.0 µmol scale syntheses were used. All syntheses were carried out Trityl-ON to prevent cleavage of the labelling group from the oligonucleotide.

HPLC Analysis and Purification of Oligonucleotides DNP-labelled using [12].

Buffer A: 0.1M NH_4OAc; *Buffer B:* 0.1M NH_4OAc/50% acetonitrile.

For oligonucleotides containing one DNP group: *Gradient A.*

For oligonucleotides containing more than one DNP group: *Gradient B.*

Polymerase Chain Reaction (PCR) Experiments.

PCR reaction mixture:

Solution	Volume
Primer 1 (10mM)	10µl
Primer 2 (10mM)	10µl
dNTP's (2mM)	10µl
10xPCR buffer	10µl
1-2ng Template DNA	2µl
Water	57.5µl
2.5U *Taq* Polymerase	0.5µl

The components of the reaction mixture were added, in the above order, to a 0.5ml sterile, siliconized eppendorf tube, and covered with 100µl of sterile mineral oil.

PCR Temperature Cycle:

The following cycle was run on a Techne PHC-3 Thermal Cycler.

(i) Denature at 94°C for 1.5min

(ii) Anneal at 55°C for 1.5min

(iii) Extend at 72°C for 2.0min

The PCR reaction mix was subjected to 30 such cycles, with a final extension at 72°C for 3 min.

Agarose Gel Electrophoresis analysis of PCR products .

Solutions:

10xTBE = 0.9M tris-borate, 25mM EDTA, pH 8.

TNE = 10mM Tris.HCl, 100mM NaCl, 1mM EDTA, pH 8.

Ethidium bromide solution = 10mgml^{-1} ethidium bromide.**[Caution:Carcinogenic]**

Loading buffer = 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll (type 400) in H_2O.

PCR products were analysed on flat-bed 1% Agarose gels, visualising the product by Ethidium Bromide staining. The gels were cast using the following mixture:

0.25g Agarose

2.5ml 10xTBE buffer

22.25ml water.

The above mixture was boiled, then after cooling to *ca.* 90°C, 2.5µl ethidium bromide solution was added and the gel cast using the hot liquid.

The gels were loaded with a mixture of 5µl of the crude PCR product and 2µl of loading buffer, and run at 100V/60mA for 15 min. Double stranded DNA is visualised as bright pink fluorescent bands in the gel when viewed on a UV transilluminator. Primers are not resolved into bands and appear as a faintly fluorescent continuum near the bottom of the gel.

Purification of PCR products.

Crude PCR product (30µl) and loading buffer (3µl) were loaded onto a 1% agarose gel. The gel was run at 120V (60mA) for 10 min, then viewed on a UV transilluminator. Wells were cut in the gel just ahead of the position of the PCR product band, and filled with 60µl of TBE. The gel was then run for 1min. and the buffer in the well was collected. This was repeated 4 times. The relative amounts of PCR product in the fractions were determined by ethidium bromide fluorescence. The combined fractions were concentrated to 100µl, and run down Sephadex G-50 spun columns (1ml) to remove the marker dyes. Ethidium bromide was removed by five butan-2-ol extractions. Presence of pure PCR product was confirmed by agarose gel electrophoresis.

Purification of Anti-DNP Monoclonal Antibody.

Pure Anti-DNP monoclonal antibody was obtained from mouse ascitic fluid using a Pierce ImmunoPure IgG Purification Kit (Protein A columns), following manufacturer's instructions. The amount of pure antibody obtained from 1ml of ascitic fluid was determined spectrophotometrically to be approximately 6.9 mg, assuming an extinction coefficient at 280nm of 1.5 $Lg^{-1}cm^{-1}$.

Dot Blot Experiments.

Solutions used:

(1) PBS/ EDTA/ EGTA: Phosphate-buffered saline solution (Sigma) containing 1mM EDTA and 1mM EGTA.

(2) PBS/ EDTA/ EGTA/Tween: Phosphate-buffered saline solution (Sigma) containing 1mM EDTA and 1mM EGTA and 0.5% Tween-20.

(3) Blocking solution: 10% solution of fat-free skimmed milk in PBS/EDTA/EGTA.

(4) K3 antibody ascitic fluid: contains approx. $7mgml^{-1}$ of monoclonal anti-DNP antibody.

(5) Avidin-HRP conjugate solution: a $0.2mgml^{-1}$ solution of avidin-horseradish peroxidase conjugate (Sigma) in PBS.

(6) DAB solution: a $0.5mgml^{-1}$ solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 50mM Tris-HCl, pH 7.6. Hydrogen peroxide (0.06% final conc.) was added just before use.

Five-fold dilutions of labelled oligonucleotides were made and 0.5µl of each solution was spotted onto nylon (Hybond N) filters (Amersham). The oligonucleotides were fixed to the membrane by UV irradiation for 5

min. The filters were blocked by incubation with blocking solution for 1h. The filters were washed with PBS/EDTA/EGTA/Tween (3x2min) and PBS/EDTA/EGTA (1x2min).

For DNP detection : The filters were incubated for 1h with a 1:200 dilution of K3 antibody ascitic fluid in PBS/EDTA/EGTA. then washed with PBS/EDTA/EGTA/Tween (3x2min) and PBS/EDTA/EGTA (1x2min). The filters were then incubated with a 1:1000 dilution of goat-anti-mouse IgG solution (SIGMA) for 1h, then washed with PBS/EDTA/EGTA/Tween (3x2min) and PBS/EDTA/EGTA (1x2min).

For Biotin detection :The filters were incubated for 1h with a 1:1000 dilution of avidin-HRP conjugate solution in PBS/EDTA/EGTA then washed with PBS/EDTA/EGTA/Tween (3x2min) and PBS/EDTA/EGTA (1x2min).

Colour reaction : The filters were incubated with DAB solution for 10-30 min. The reaction was stopped by washing the filter several times with water.

Capture and Detection of DNP-Biotin Labelled Oligonucleotides.

Solutions used as for Dot Blot Experiments.

Fixing of Avidin to Nitrocellulose and blocking : A nitrocellulose membrane (Amersham Hybond-C) was pre-wetted with PBS. A solution of avidin ($0.1 mg ml^{-1}$) in PBS was spotted onto the membrane in 1ml aliquots (gives 0.1μg of avidin/spot). The membrane was incubated at 4°C for 4h, then washed once with blocking solution. The membrane was incubated with blocking solution for 1h, then washed with PBS/Tween (3x5min).

Oligonucleotide capture : The membrane was cut into strips and each strip was placed in a sealed polythene bag containing 3.3ml

PBS/EDTA/EGTA and 0.7ml of a 1mM solution of oligonucleotide in PBS/EDTA/EGTA (gives an oligonucleotide conc. of 0.175μM). The strips were incubated in these bags for 1h, then washed successively with PBS/EDTA/EGTA (3x5min), PBS/Tween (3x5min) and PBS (3x5min).
Oligonucleotide detection :The strips were incubated with a 1:200 dilution of K3 antibody ascitic fluid in PBS/EDTA/EGTA for 1h. then washed with PBS/EDTA/EGTA (3x5min). The filters were then incubated with a 1:1000 dilution of goat-anti-mouse IgG solution (Sigma) for 1h, then washed with PBS/EDTA/EGTA (3x5min).
Colour reaction : The filters were incubated with DAB solution for 30 min. The reaction was stopped by washing the filter several times with water.

Multiple Hydroxyl Phosphoramidite.

1,6-bis(4,4'-Dimethoxytrityloxy)hexan-2-ol [13].

1,2,6-Trihydroxyhexane (1eq; 1.00g; 7.45mmol) was dissolved in anhydrous pyridine (25ml) and to this solution was added 4,4'-dimethoxytrityl chloride (2.2eq; 0.016mol; 5.56g). After stirring for 24h at 20°C the reaction was quenched with methanol (2ml) and solvents removed by evaporation *in vacuo*. The residue was dissolved in dichloromethane and washed with sat $NaHCO_3$ (50ml) and sat. KCl (3 x 50ml). The organic phase was dried (Na_2SO_4), filtered and solvents removed by evaporation *in vacuo* . The residue was purified by wet-flash silica gel chromatography on a column which had been equilibrated with hexane containing 10% ethyl acetate and 1% triethylamine eluting with a gradient of 10-50% ethyl acetate in hexane, to give the *title compound* **[13]** as a white foam (1.920g; 35%). R_f 0.55 (solvents J and B), R_f 0.83 (solvent H); ν_{max}(nujol)/cm^{-1} 3550 (m, OH), 2720(m), 2020 (w), 1720

(m), 1610 (m, aromatic), 1580(m, aromatic), 1300(s), 1250(s), 1170(m) 1155(w), 1115(w), 1075(w), 1035 (w), 970 (w), 915(w), 825(m), 790(w), 770(w), 750 (m), 720(s). δ_H(200.130MHz; $CDCl_3$) 1.31-1.75 (6H,m,CH_2), 2.25-2.45 (1H, brs,OH), 2.98-3.22 (5H, m, CH, CH_2), 3.77 (6H, s, OCH_3), 3.78 (6H, s, OCH_3), 6.78-6.87 (8H, m, DMT-CH), 7.19-7.48 (18H, m, DMT-CH); δ_C(50.32MHz; $CDCl_3$) 22.14 (CH_2), 29.87 (CH_2), 33.07 (CH_2), 55.00 (4xDMT-CH_3), 63.02 (CH_2), 67.39 (CH_2), 70.76 (CH), 85.47 (DMT-C), 85.87 (DMT-C), 112.79 (4xDMT-CH), 112.96 (4xDMT-CH), 126.38 (DMT-CH), 126.64 (DMT-CH), 127.52 (2xDMT-CH), 127.67 (2xDMT-CH), 127.99 (4xDMT-CH), 129.85 (8xDMT-CH), 135.89 (2xDMT-C), 136.51 (2xDMT-C), 144.71 (DMT-C), 145.22 (DMT-C), 158.12 (2xDMT-C), 158.31 (2xDMT-C). f.a.b. ms *m/z* 738.35561 [M^+, $C_{48}H_{50}O_7$ requires 738.35563].

2-Cyanoethyl [1,6-bis(4,4'-dimethoxytrityloxy) hex-2-yl] N,N-diisopropylamino phosphoramidite [14].

To a solution of compound **[13]** (1eq; 1.084g; 1.47mmol) in anhydrous tetrahydrofuran (10ml) was added N,N-diisopropylethylamine (4eq; 5.88mmol; 0.76g; 1.02ml) and 2-cyanoethyl N,N-di-isopropyl-phosphoramidochloridite (1eq; 1.47mmol; 0.348g; 0.33ml). After 1h the reaction was diluted with ethyl acetate (100ml). The resulting solution was washed with sat. aqueous KCl (4 x 50ml). The organic phase was dried ($MgSO_4$), filtered and solvents were removed by evaporation *in vacuo* . A silica gel column was pre-equilibrated with hexane containing 20% ethyl acetate and 1% triethylamine and the residue was then purified on this column, eluting with a gradient of 20-30% ethyl acetate in hexane, to give the *title compound* **[14]** as a clear oil (1.217g; 89%). R_f 0.21 (solvent N), R_f 0.65 (solvent B); δ_P(81.02 MHz; CH_3CN solvent;

$(CD_3)_2CO$ int) 147.57(s), 147.74 (s). f.a.b. ms *m/z* 939.47135 $[(M+H)^+$, $C_{57}H_{68}N_2O_8P$ requires 939.4713].

Oligonucleotide synthesis using [14].

A 0.15M solution of **[14]** in anhydrous acetonitrile was used for oligonucleotide synthesis. Single addition biotin and DNP phosphoramidites were used as 0.15M solutions in anhydrous acetonitrile. Modifications to the standard synthesis cycle for 0.2 μmol syntheses were as follows:

(i) Double delivery of Base and Base+tetrazole to column during coupling step for both **[14]** and biotin or DNP phosphoramidites.

(ii) Coupling wait step increased to 5min. for additions of both **[14]** and biotin or DNP phosphoramidites.

HPLC Analysis and Purification.

Buffer A: 0.1M NH_4OAc; *Buffer B:* 0.1M NH_4OAc/50% acetonitrile. Gradient B.

Biotin Derivatives.

$N^{1'}$-Benzoylbiotin-2-nitrophenyl ester [15]

Biotin-2-nitrophenyl ester (1eq; 1.5g; 4.11mmol) was dried by evaporation of anhydrous pyridine (3 x 15ml), then dissolved in anhydrous pyridine (20ml), and cooled to 0°C. Benzoyl chloride (2eq; 8.22mmol; 1.15g; 0.95ml) was added dropwise with stirring. The reaction was allowed to warm to room temperature and tlc indicated that all the starting material was consumed after 90 min. The reaction mixture was poured into sat. aqueous $NaHCO_3$, extracted with dichloromethane and the organic phase was washed twice with sat. aqueous KCl, then dried

(Na_2SO_4), filtered and the solvent was removed by evaporation *in vacuo* to give a yellow foam. The product was purified by wet-flash column chromatography, eluting with a gradient of 0-20% ethyl acetate in dichloromethane, to give the *title compound* **[15]** as a white foam (1.36g; 70%). R_f 0.63 (solvent C), 0.43 (solvent H); δ_H(200.130MHz; DMSO-d6) 1.45-1.79 (6H, m, 6-, 7-, 8-H), 2.68 (2H, t, 9-H, J_{8-9}=7.2Hz), 2.89-3.12 (2H, m, 2-H), 3.24-3.30 (1H, m, 5-H), 4.25 (1H, dd, H-4, J_{3-4}=7.7Hz, J_{4-5}=4.5Hz), 5.07 (1H, m, H-3), 7.34-7.60 (8H, m, H-3', Bz CH, nitrophenyl 4-H, 6H), 7.80-7.88 (1H, dt, nitrophenyl 5-H, J_{3-5}=1.6Hz, $J_{5-4,6}$=7.7Hz), 8.13-8.18 (1H, dd, nitrophenyl 3-H, J_{3-5}=1.6Hz, J_{3-4}=8.1Hz); δ_C(50.32 MHz; $CDCl_3$) 23.87 (CH_2), 27.59 (CH_2), 27.96 (CH_2), 33.46 (CH_2), 38.10 (CH_2), 54.98 (CH), 57.52 (CH), 62.17 (CH), 125.04 (CH), 125.65 (CH), 126.54 (CH), 127.34 (CH), 128.66 (CH), 131.28 (CH), 134.62 (CH), 134.72 (CH), 141.64(C), 143.87(C), 156.56(C), 169.69(C), 170.96(C). f.a.b. ms m/z 470.13855 [$(M+H)^+$, $C_{23}H_{24}N_3O_6S$ requires 470.13857].

(N^5-($N^{1'}$-Benzoylbiotinyl))-5-amino-1-hydroxy-3-oxa-pentane [16]

N1'-Benzoylbiotin-2-nitrophenyl ester **[15]** (1eq; 0.7g; 1.49mmol) was dissolved in dichloromethane (20ml) and to this solution was added triethylamine (0.2ml) and 2-(2-aminoethoxy)ethanol (1eq; 1.49mmol; 0.15g). The solution immediately turned bright yellow due to the liberated 2-nitrophenol. The reaction was complete after 2hr at 20°C whereupon the solution was evaporated to dryness and coevaporated with toluene (2x7ml) then ethanol (7ml). The product was purified by wet-flash chromatography eluting with a gradient of 1-2% methanol in dichloromethane, to give the *title compound* **[16]** as a white solid (0.586g; 90%). R_f 0.25 (solvent C); δ_H(200.130MHz; DMSO-d6/D_2O) 1.26-1.59

(6H, m, 6-, 7-, 8-H), 2.09 (2H, t, 9-H, J_{8-9}=7.2Hz), 2.87-2.96 (2H, m, 2-H), 3.15-3.26 (3H, m, 5-H, CH_2), 3.37-3.54 (6H, m, CH_2), 4.22 (1H, dd, H-4, J_{3-4}=7.8Hz, J_{4-5}=4.6Hz), 5.01-5.06 (1H, m, H-3), 7.27-7.47 (5H, m, Ar). f.a.b. ms *m/z* 436.19062 [$(M+H)^+$, $C_{21}H_{30}N_3O_5S$ requires 436.19060].

1-[(N^5-($N^{1'}$-Benzoylbiotinyl))-5-amino-3-oxa-pentyl]-2-cyanoethyl-N,N-diisopropylamino phosphoramidite [17]

(N5-(N1'-Benzoylbiotinyl))-5-amino-1-hydroxy-3-oxa-pentane **[16]** (1eq; 0.54g; 1.24mmol) was dissolved in anhydrous tetrahydrofuran (15ml). To this solution was added N, N-diisopropylethylamine (4eq; 4.97mmol; 0.64g; 0.86ml) and 2-cyanoethyl N,N-di-isopropylphosphoramido-chloridite (1eq; 1.24mmol; 0.29g; 0.28ml). The reaction was incomplete after 1hr and a further 0.3 eq (0.09ml) of 2-cyanoethyl N,N-di-isopropyl-phosphoramidochloridite was added. After a further hour the reaction was quenched with anhydrous ethanol (5ml), and the solution was evaporated to an oil which was dissolved in dichloromethane and washed once with sat. aqueous $NaHCO_3$ and twice with sat. aqueous KCl. The organic phase was dried (Na_2SO_4), filtered and the solvent evaporated *in vacuo* . The product was purified by wet flash chromatography eluting with dichloromethane containing 0-5% methanol and 1% triethylamine. The purified product was dissolved in dichloromethane and precipitated from anhydrous hexane at 20°C. The precipitate was allowed to settle and the supernatant was removed *via* a double ended needle. The solid residue was dissolved in dichloromethane and evaporated *in vacuo* to give the *title compound* **[17]** as a white foam (0.346g; 43%). The product was found by ^{31}P-NMR to be contaminated with *ca.* 10% of the corresponding phosphonate. This did not appear to interfere with

coupling during oligonucleotide synthesis. R_f 0.48 (solvent C); δp(80MHz; CH_2Cl_2solvent; $(CD_3)_2CO$ int.) 148.347, 9.019. f.a.b. ms *m/z* 636.29846 [$(M+H)^+$, $C_{30}H_{47}N_5O_6PS$ requires 636.29845]

1-Phthalimido-8-tetrahydropyranyloxyoctane [18]

1-Bromo-8-tetrahydropyranyloxyoctane (1eq; 10g; 34mmol) was dissolved in anhydrous dimethylformamide (50ml) and to this solution was added potassium phthalimide (1.1eq; 37.5mmol; 6.95g). The reaction was stirred at 20°C for 24h, then evaporated to dryness. The residue was dissolved in ethyl acetate and the precipitate of KBr and excess potassium phthalimide was filtered off. The resulting solution was washed once with water, dried (Na_2SO_4), filtered and the solvent was removed by evaporation *in vacuo* . The product was purified by wet-flash chromatography, eluting with a gradient of 10-20% ethyl acetate in hexane, to give the *title compound* **[18]** as a clear oil, which solidified on standing (11.07g;91%). R_f 0.74 (solvent I), 0.67 (solvent J); ν_{max}(nujol)/cm^{-1} 1770 (m, cyclic imide), 1715 (s, cyclic imide) , 1390 (s), 1255 (w), 1200 (w), 1185 (w), 1170 (w), 1135 (m), 1120 (m), 1075 (s), 1065 (s), 1030 (s), 990 (w), 965 (w), 940 (w), 905 (w), 865 (m), 815 (w), 790 (w), 715 (s), 690 (w). δ_H(200.130MHz; $CDCl_3$) 1.25-1.76 (18H, m, CH_2), 3.23-3.83 (6H, m, NCH_2, OCH_2), 4.49 (1H, t, THP-CH, J=3.1Hz), 7.61-7.78 (4H, m, Ar-H); δ_C(50.32MHz; $CDCl_3$) 19.46 (CH_2), 25.29 (CH_2), 25.95 (CH_2), 26.59 (CH_2), 28.37 (CH_2), 28.92 (CH_2), 29.10 (CH_2), 29.48 (CH_2), 30.55 (CH_2), 37.81 (CH_2), 62.05 (CH_2), 67.36 (CH_2), 98.57 (THP-CH), 122.92 (2xAr CH), 131.95 (2xAr C), 133.62 (2xAr CH), 168.20 (2xCO). f.a.b. ms *m/z* 360.21745 [$(M+H)^+$, $C_{21}H_{30}NO_4$ requires 360.21747].

1-Amino-8-tetrahydropyranyloxyoctane [19]

1-Phthalimido-8-tetrahydropyranyloxyoctane **[18]** (1eq; 5g; 0.014mol) was dissolved in methanol (20ml). To this solution was added hydrazine hydrate (1.1eq; 0.015mol; 0.77g; 0.74ml). After 5h the reaction was evaporated to dryness and the residue was dissolved in diethyl ether and extracted once with 2M aqueous NaOH. The organic phase was dried (Na_2SO_4), filtered and the solvent evaporated to give an oil which solidified on standing. The product was purified by kugelrohr distillation, to give the *title compound* **[19]** as a clear liquid (2.2g; 69%). b.p. 180°C (at 0.5mbar); R_f 0.45 (solvent K); ν_{max}(film)/cm^{-1} 3360 (m, NH_2), 3295 (m, NH_2), 2920 (s, aliphatic), 2845 (s, aliphatic), 1575 (m), 1460 (m), 1440 (m), 1380 (m), 1360 (m), 1350 (m), 1320 (m), 1280 (w), 1260 (w), 1200 (m), 1180 (m), 1160 (m), 1135 (s), 1120 (s), 1075 (s), 1030 (s), 985 (m), 970 (m), 905 (m), 865 (m), 815 (m), 720 (w); δ_H(200.130MHz; $CDCl_3$) 1.20-1.73 (18H, m, CH_2), 2.56 (2H, t, NCH_2, J=6.7Hz), 3.20-3.81 (4H, m, OCH_2), 4.46 (1H, t, THP-CH, J=3.2Hz); δ_C(50.320MHz; $CDCl_3$) 19.33, 25.15, 25.85, 26.49, 29.09(2xCH_2), 29.38, 30.42, 33.48, 41.88, 61.93, 67.26, , 76.25, 76.89, 77.53, 98.45 (CH). f.a.b. ms *m/z* 230.21197 [$(M+H)^+$, $C_{13}H_{27}NO_2$ requires 230.21199].

N-(N$^{1'}$-Benzoylbiotinyl)-1-amino-8-tetrahydropyranyloxyoctane [20].

Compound **[19]** (1eq; 0.94g; 2mmol) was dissolved in anhydrous tetrahydrofuran (20ml). To this solution was added compound **[15]** (1eq; 2mmol; 0.46g) and triethylamine (1eq; 2mmol; 0.20g; 0.28ml). After 30min the reaction was evaporated to dryness, then coevaporated with ethanol (10ml) then toluene (10ml). The residue was dissolved in dichloromethane and this solution was washed with sat. aqueous $NaHCO_3$(50ml) then sat. aqueous KCl (50ml). The aqueous washings

were extracted twice with dichloromethane, and the combined organic phase was dried (Na_2SO_4), filtered and the solvent evaporated *in vacuo* . The product was purified by wet-flash chromatography, eluting with dichloromethane containing 2-10% methanol. The title compound **[20]** was obtained as a white solid (1.069g; 96%). R_f 0.60 (solvent C); δ_H(200.130MHz; $CDCl_3$) 1.25-1.76 (24H, m, 6-, 7-, 8-H, octyl CH_2), 2.05 (2H, m, 9-H), 2.91-3.14 (5H, m, 2-H, 5-H, NCH_2), 3.26-3.87 (4H, m, OCH_2), 4.10 (1H, dd, 4-H, $J_{3\text{-}4}$=7.6Hz, $J_{4\text{-}5}$=4.6Hz), 4.50 (1H, m, THP-CH), 5.10 (1H, m, 3-H), 5.78 (1H, m, CONH), 7.00 (1H, s, 3'-H), 7.25-7.55 (5H, m, Ar-H); δ_C(50.32MHz; $CDCl_3$) 19.57(CH_2), 25.16(CH_2), 25.30(CH_2), 25.98(CH_2), 26.69(CH_2), 27.39(CH_2), 27.68(CH_2), 29.05(CH_2), 29.19(CH_2), 29.41(CH_2), 29.53(CH_2), 30.62(CH_2), 35.54(CH_2), 38.02(CH_2), 39.35(CH_2), 54.98(5-C), 57.32(4-C), 62.15(3-C), 62.26(THP-CH_2), 67.46(THP-CH_2), 98.76(THP-CH), 127.31(benzoyl 3-&5-C), 128.61(benzoyl 2-&6-C), 131.24(benzoyl 4-C), 134.59 (benzoyl 1-C), 156.20(benzoyl CO), 169.76 (2'-C), 172.55(10-C). f.a.b. ms *m/z* 560.31579 [$(M+H)^+$, $C_{30}H_{46}N_3O_5S$ requires 560.31580]

N^1-($N^{1'}$-Benzoylbiotinyl)-1-aminooctan-8-ol [21].

To a solution of compound **[20]** (1eq; 0.9g; 1.61mmol) in methanol (25ml) was added DOWEX H^+ form (30g). After 3h the DOWEX was filtered-off and washed with methanol (150ml). The combined filtrate was evaporated to dryness and the residue was purified by wet-flash column chromatography, eluting with 10% methanol in dichloromethane, to give the title compound **[21]** as a white solid (0.712g; 93%). R_f 0.36 (solvent C); δ_H(200.130MHz; $CDCl_3$) 1.03-1.55 (18H, m, 6-, 7-, 8-H, octyl CH_2), 1.88 (2H, t, 9-H, J=7.0Hz), 2.69-3.00 (5H, m, 2-H, 5-H, CH_2OH), 3.21-3.29 (2H, m, NCH_2), 3.45-3.47 (1H, m, OH), 3.89-3.95

(1H, m, H-4), 4.83-4.90 (1H, m, 3-H), 6.66 (1H, t, CONH, J=5.3Hz), 7.04-7.40 (6H, m, Ar-H, 3'-H); δ_C(50.32MHz; $CDCl_3$) 25.22(CH_2), 25.39(CH_2), 26.48(CH_2), 27.54(CH_2), 27.85(CH_2), 28.86(CH_2), 28.96(CH_2), 29.13(CH_2), 32.37(CH_2), 35.34(CH_2), 37.69(CH_2), 38.96(CH_2), 55.04(5-CH), 57.18(4-CH), 61.73(CH_2), 61.74(CH), 127.09(benzoyl 3-&5-C), 128.30(benzoyl 2-&6-C), 130.82(benzoyl 4-C), 134.75 (benzoyl 1-C), 155.65(benzoyl CO), 169.55 (2'-C), 172.76(10-C). f.a.b. ms *m/z* 476.25828 [$(M+H)^+$, $C_{25}H_{38}N_3O_4S$ requires 476.25829].

8-[N^1-($N^{1'}$-Benzoylbiotinyl)-1-aminooctyl]-2-cyanoethyl-N,N-diisopropylamino phosphoramidite [22].

Compound **[21]** (1eq; 0.40g; 0.842mmol) was dissolved in anhydrous tetrahydrofuran (5ml). To this solution was added N, N-diisopropylethylamine (4eq; 3.37mmol; 0.44g; 0.59ml) and 2-cyanoethyl N,N-di-isopropylphosphoramidochloridite (1eq; 0.84mmol; 0.19g; 0.19ml). The reaction which was complete after 5min was quenched by the addition of anhydrous ethanol (1ml). Dichloromethane was added and the resulting solution was washed sat. aqueous $NaHCO_3$ (50ml) and sat. aqueous KCl (2x50ml). The organic phase was dried (Na_2SO_4), filtered and the solvent was removed by evaporation *in vacuo* . A silica gel column was pre-equilibrated with ethyl acetate containing 20% acetonitrile and 1% triethylamine and the product was purified on this column, eluting with ethyl acetate containing 20-50% acetonitrile, to give the *title compound* **[22]** as a white solid (0.251g; 44%). R_f 0.53 (solvent L); δ_P(80MHz; ethyl acetate solvent; $(CD_3)_2CO$ int) 146.866 (s). f.a.b. ms *m/z* 676.36612 [$(M+H)^+$, $C_{34}H_{55}N_5O_5PS$ requires 676.36613].

Large scale synthesis and characterisation of [22]p$(T_p)_4$T.

This sequence was synthesised in order to fully characterise the group which was introduced onto the 5'-end of the oligonucleotide by **[22]**, and to demonstrate that full deprotection of the biotin ring had occurred. A total of six 1.0 μmol scale syntheses were carried out and the desired product was purified by HPLC and desalted on a Sephadex G-10 column. After lyophilization the biotinylated oligonucleotide was obtained in 10.1mg yield. The entire sample was dissolved in 0.5ml of H_2O:D_2O / 9:1 (v/v) and analysed by 600MHz ^{1}H-NMR using Correlated Spectroscopy (COSY).

δ_H(599.945MHz; H_2O:D_2O/9:1) 1.15-1.22 (8H, m, octyl 3-,4-,5-,6-H), 1.33-1.43 (4H, m, octyl 2-H, biotin 7-H), 1.51-1.72 (6H, m, octyl 7-H, biotin 6-,8-H), 1.85-1.93 (m, thymidine 5-H, acetate), 2.22-2.37 (8H, m, biotin 9-H, thymidine 2'-,2"-H), 2.49-2.54 (4H, m, thymidine 2'-,2"-H), 2.76 (1H, d, biotin pro-R 2-H, J_{gem}=13.1Hz), 2.96 (1H, dd, biotin pro-S 2-H, J_{gem}=13.1Hz, $J_{2\text{-}3}$=5Hz), 3.09-3.17 (2H, m, octyl 1-H), 3.26-3.29 (1H, m, biotin 5-H), 3.79-3.85 (2H, m, octyl 8-H), 4.03-4.15 (11H, m, thymidine 4'-,5'-,5"-H), 4.30 (3H, m, thymidine 4'-H), 4.35 (1H, m, thymidine 4'-H), 4.39 (1H, dd, biotin 4-H, $J_{3\text{-}4}$=7.9Hz, $J_{4\text{-}5}$=4.6Hz), 4.57-4.59 (2H, m, biotin 3-H, thymidine 3'-H), 4.71-4.84 (thymidine 3'-H masked by H_2O signal), 6.23-6.31 (5H, m, thymidine 1'-H), 7.65 (1H, s, thymidine 6-H), 7.66 (1H, s, thymidine 6-H), 7.68 (1H, s, thymidine 6-H), 7.71 (1H, s, thymidine 6-H), 7.74 (1H, s, thymidine 6-H), 7.90 (1H, t, CONH, J=5.7Hz).

D. REFERENCES.

(1) Watson, J.D.; Witkowski, J.; Gilman, M.; Zoller, M. '*Recombinant DNA*", Scientific American Books, (1992).

(2) Keller, G.H.; Manak, M.M. (1989) '*DNA Probes.*' Stockton Press.

(3) Brigati, D.J.; Myerson, D.; Leary, J.J.; Spalholz, B.; Travis, S.Z.; Fong, C.K.Y.; Hsiung, G.D.; Ward, D.C. (1983) *Virology*, **126**, 32-50.

(4) Muhlegger, K.; Huber, E.; vdEltz, H.; Ruger, R.; Kessler, C. (1990) *Biol. Chem. Hoppe Seyler,* **371**, 953-965.

(5) Smith, L.M.; Fung, S.; Hunkapiller, M.W.; Hood, L.E. (1985) *Nucleic Acids Res.* **13**, 2399-2412.

(6) Smith, L.M.; Sanders, J.Z.; Kaiser, R.J.; Hughes, P.; Dod, C.; Connell, C.R.; Heiner, C.; Kent, S.B.H.; Hood, L.E. (1986) *Nature* , **321**, 674-678.

(7) Smith, L.M.; Kaiser, R.J.; Sanders, J.Z.; Hood, L.E. (1987) *Methods in Enzymology,* **155**, 260-301.

(8) Kaiser, R.J.; MacKellar, S.L.; Vinayak, R.S.; Sanders, J.Z.; Saavedra, R.A.; Hood, L.E. (1989) *Nucleic Acids Res.* **17**, 6087-6102.

(9) *ABI User Bulletin, DNA Sequencer.* (1989), **No.11**, Applied Biosystems Inc.

(10) Ansorge, W.; Sproat, B.; Stegemann, J. (1987) *Nucleic Acids Res.* **15**, 4593-4602.

(11) Korenberg, J.R.; Yang-Feng, T.; Schreck, R.; Chen, X.N. (1992) *Trends in Biotechnology*, **10**, 27-32.

(12) Jablonski, E.; Moomaw, E.W.; Tullis, R.H.; Ruth, J.L. (1986) *Nucleic Acids Res.* **14**, 6115-6128.

(13) Renz, M.; Kurz, C. (1984) *Nucleic Acids Res.* **12**, 3435-3444.

(14) Saiki, R.K.; Chang, C.A.; Levenson, C.H.; Warren, T.C.; Boehm, C.D.; Kazazian, H.H.; Erlich, H.A. (1988) *New Eng. J. Medicine,* **319**, 537-541.

(15) Urdea, M.S.; Warner, B.D.; Running, J.A.; Stempien, M.; Clyne, J.; Horn, T. (1988) *Nucleic Acids Res.* **16**, 4937-4956.

(16) Blake, M.S.; Johnston, K.H.; Russel-Jones, G.J.; Gotschlich, E.C. (1984) *Analytical Biochem.* **136**, 175-179.

(17) Horwitz, J.P.; Chuo, J.; Noel, M.; Donatti, J.T.; Freisler, J. (1966) *J. Med. Chem.* **9**, 447.

(18) Tsou, K.C.; Cheng, C.S.; Nachlas, M.M.; Seligman, A.M. (1956) *J. Am. Chem. Soc.* **78**, 6139-6144.

(19) Nineham, A.W. (1955) *Chem. Rev.* **55**, 355-483.

(20) Langer-Safer, P.R.; Levine, M.; Ward, D.C. (1982) *Proc. Natl. Acad. Sci. USA,* **79,** 4381-4385.

(21) Durrant, I. (1990) *Nature*, **346**, 297-298.

(22) Matthews, J.A.; Batki, A.; Hynds, C.; Kricka, L.J.; (1985) *Analytical Biochem.* **151**, 205-209.

(23) Huntress, E.H.; Stanley, L.N.; Parker, A.S. (1934) *J. Am. Chem. Soc.* **56**, 241-242.

(24) Roswell, D.F.; White, E.H. (1978) *Methods in Enzymology,* **LVII**, 409-423.

(25) Schaap, A.P.; Sandison, M.D.; Handley, R.S. (1987) *Tetrahedron Lett.* **28**, 1159-1162.

(26) Chollet, A.; Kawashima, E.H. (1985) *Nucleic Acids Res.* **13**, 1529-1541.

(27) Misiura, K.; Durrant, I.; Evans, M.R.; Gait, M.J. (1990) *Nucleic Acids Res.* **18**, 4345-4354.

(28) Seibl, R.; Holtke, H.J.; Ruger, R.; Meindl, A.; Zachau, H.G.; Rasshofer, R.; Roggendorf, M.; Wolf, H.; Arnold, N.; Wienberg, J.; Kessler, C. (1990) *Biol. Chem. Hoppe Seyler,* **371**, 939-951.

(29) Muhlegger, K.; Batz, H.G.; Bohm, H.; vdEltz, H.; Holtke, H.J.; Kessler, C. (1989) *Nucleosides and Nucleotides,* **8**, 1161-1163.

(30) Keller, G.H.; Cumming, C.U.; Huang, D.P.; Manak, M.M.; Ting, R. (1988) *Analytical Biochem.* **170**, 441-450.

(31) Vincent, C.; Tchen, P.; Cohen-Solal, M.; Kourilsky, P. (1982) *Nucleic Acids Res.* **10**, 6787-6796.

(32) Keller, G.H.; Huang, D.P.; Manak, M.M. (1989) *Analytical Biochem.* **177**, 392-395.

(33) Tchen, P. *DNA*, **4**, 93.

(34) Tchen, P.; Fuchs, R.R.P.; Sage, E.; Leng, M. (1984) *Proc. Natl. Acad. Sci. USA,* **81**, 3466-3470.

(35) Kumar, A.; Tchen, P.; Roullet, F.; Cohen, J. (1988) *Analytical Biochem.* **169**, 376-382.

(36) Langer, P.R.; Waldrop, A.A.; Ward, D.C. (1981) *Proc. Natl. Acad. Sci. USA,* **78,** 6633-6637.

(37) Leary, J.J.; Brigati, D.J.; Ward, D.C. (1983) *Proc. Natl. Acad. Sci. USA,* **80,** 4045-4049.

(38) Cosstick, R.; McLaughlin, L.W.; Eckstein, F. (1984) *Nucleic Acids Res.* **12**, 1791-1810.

(39) Sinha, N.D.; Striepeke, S. in 'Oligonucleotides and Analogues-a Practical Approach', Eckstein, F. Ed. IRL Press, Oxford. (1991) 185-210.

(40) *ABI User Bulletin, DNA synthesizer* (1988), **No.49**, Applied Biosystems Inc.

(41) Boehringer-Mannhein Digoxigenin product information.

(42) Connolly, B.A. (1987) *Nucleic Acids Res.* **15**, 3131-3139.

(43) Tanaka, T; Tamatsukuri, S.; Ikehara, M. (1987) *Tetrahedron Lett.* **28**, 2611-2614.

(44) Coull, J.M.; Weith, H.L.; Bischoff, R. (1986) *Tetrahedron Lett.* **27**, 3991-3994.

(45) Agrawal, S.; Christodoulou, C.; Gait, M.J. (1986) *Nucleic Acids Res.* **14**, 6227-6245.

(46) Sinha, N.D.; Cook, R.M. (1988) *Nucleic Acids Res.* **16**, 2659-2669.

(47) Bannwarth, W.; Schmidt, D.; Stallard, R.L.; Horning, C.; Knorr, R.; Muller, F. (1988) *Helv. Chim. Acta*, **71**, 2085-2099.

(48) Agrawal, S. (1989) *Tetrahedron Lett.*, **30**, 7025-7028.

(49) Nelson, P.S.; Sherman-Gold, R.; Leon, R. (1989) *Nucleic Acids Res.* **17**, 7179-7186.

(50) Nelson, P.S.; Frye, R.A.; Liu, E. (1989) *Nucleic Acids Res.* **17**, 7187-7194.

(51) Sproat, B.S.; Beijer, B.; Rider, P. (1987) *Nucleic Acids Res.* **15**, 6181-6196.

(52) Manoharan, M.; Guinosso, C.J.; Cook, P.D. (1991) *Tetrahedron Lett.* **32**, 7171-7174.

(53) Kansal, V.K.; Huynh-Dinh, T.; Igolen, J. (1988) *Tetrahedron Lett.* **29**, 5537-5540.

(54) Chu, B.C.F.; Wahl, G.M.; Orgel, L.E. (1983) *Nucleic Acids Res.* **11**, 6513-6529.

(55) Agrawal, S.; Tang, J.Y. (1990) *Tetrahedron Lett.*, **31**, 1543-1546.

(56) Agrawal, S.; Zamecnik, P.C. (1990)*Nucleic Acids Res.* **18**, 5419-5423.

(57) Tang, J.Y.; Agrawal, S. (1990) *Nucleic Acids Res.* **18**, 6461.

(58) Wachter, L.; Jablonski, J.A.; Ramachandran, K.L. (1986) *Nucleic Acids Res.* **14**, 7985-7994.

(59) Haralambidis, J.; Angus, K.; Pownall, S.; Duncan, L.; Chai, M.; Tregear, G.W. (1990) *Nucleic Acids Res.* **18**, 501-505.

(60) Haralambidis, J.; Duncan, L.; Angus, K.; Tregear, G.W. (1990) *Nucleic Acids Res.* **18**, 493-499.

(61) Jablonski, E.; Ruth, J.L. (1986) *DNA*, **5**, 89.

(62) Ruth, J.L. in 'Oligonucleotides and Analogues-a Practical Approach', Eckstein, F. Ed. IRL Press, Oxford. (1991) 255-282.

(63) Gibson, K.J.; Benkovic, S.J. (1987) *Nucleic Acids Res.* **15**, 6455-6467.

(64) Cook, A.F.; Vuocolo, E.; Brakel, C.L. (1988) *Nucleic Acids Res.* **16**, 4077-4095.

(65) Kierzek, J.; Markiewicz, W. (1987) *Nucleosides and Nucleotides*, **6**, 403-405.

(66) Sproat, B.S.; Lamond, A.I.; Beijer, B.; Neuner, P.; Ryder, U. (1989) *Nucleic Acids Res.* **17**, 3373-3386.

(67) Telser, J.; Cruickshank, K.A.; Morrison, L.E.; Netzel, T.L. (1989) *J. Am. Chem. Soc.* **111**, 6966-6976.

(68) Telser, J.; Cruickshank, K.A.; Morrison, L.E.; Netzel, T.L.; Chan, C. (1989) *J. Am. Chem. Soc.* **111**, 7226-7232.

(69) Bonfils, E.; Thuong, N.T. (1991) *Tetrahedron Lett.* **32**, 3053-3056.

(70) Gupta, K.C.; Sharma, P.; Sathyanarayana, ; Kumar, P. (1990) *Tetrahedron Lett.* **31**, 2471-2474.

(71) Li, P.; Medon, P.P.; Skingle, D.C.; Lanser, J.A.; Symons, R.H. (1987) *Nucleic Acids Res.* **15**, 5275-5287.

(72) Zuckermann, R.; Corey, D.; Schultz, P. (1987) *Nucleic Acids Res.* **15**, 5305-5321.

(73) Eshaghpour, H.; Soll, D.; Crothers, D.M. (1979) *Nucleic Acids Res.* **7**, 1485-1495.

(74) Connolly, B.A.; Rider, P. (1985) *Nucleic Acids Res.* **13**, 4485-4502.

(75) Sproat, B.S.; Beijer, B.; Rider, P.; Neuner, P. (1987) *Nucleic Acids Res.* **15**, 4837-4848.

(76) Kumar, A.; Advani, S.; Dawar, H.; Talwar, G.P. (1991) *Nucleic Acids Res.* **19**, 4561.

(77) Gaur, R.K.; Sharma, P.; Gupta, K.C. (1989) *Nucleic Acids Res.* **17**, 4404.

(78) Murakami, A.; Tada, J.; Yamagata, K.; Takano, J. (1989) *Nucleic Acids Res.* **17**, 5587-5595.

(79) Fidanza, J.A.; McLaughlin, L.W. (1989) *J. Am. Chem. Soc.* **111**, 9117-9119.

(80) Conway, N.E.; Fidanza, J.A.; O'Donnell, M.A.J.; Narekian, N.D.; Ozaki, H.; McLaughlin, L.W. in 'Oligonucleotides and Analogues-a Practical Approach', Eckstein, F. Ed. IRL Press, Oxford. (1991) 211-239.

(81) Brown, T.; Brown, D.J.S. in 'Oligonucleotides and Analogues-a Practical Approach', Eckstein, F. Ed. IRL Press, Oxford. (1991) 1-24.

(82) Forster, A.C.; McInnes, J.L.; Skingle, D.C.; Symons, R.H. (1985) *Nucleic Acids Res.* **13**, 745-761.

(83) Rothenberg, J.M.; Wilchek, M. (1988) *Nucleic Acids Res.* **16**, 7197.

(84) Ruth, J.L. (1984) *DNA*, **3**, 123.

(85) Alves, A.M.; Holland, D.; Edge, M.D. (1989) *Tetrahedron Lett.* **30**, 3089-3092.

(86) Cocuzza, A.J. (1989) *Tetrahedron Lett.* **30**, 6287-6290.

(87) Pon, R.T. (1991) *Tetrahedron Lett.* **32**, 1715-1718.

(88) Pritchard, C. (1990), *PhD. Thesis*, Edinburgh University.

(89) Schubert, F.; Ahlert, K.; Cech, D.: Rosenthal, A. (1990) *Nucleic Acids Res.* **18**, 3427.

(90) Bannwarth, W.; Schmidt, D. (1989) *Tetrahedron Lett.* **30**, 1513-1516.

(91) Roget, A.; Bazin, H.; Teoule, R. (1989) *Nucleic Acids Res.* **17**, 7643-7651.

(92) Schulhof, J.C.; Molko, D.; Teoule, R. (1987) *Nucleic Acids Res.* **15**, 397-416.

(93) Pieles, U.; Sproat, B.S.; Lamm, G.M. (1990) *Nucleic Acids Res.* **18**, 4355-4360.

(94) Thiesen, P.; McCollum, C.; Upadhya, K.; Jacobson, K.; Vu, H.; Andrus, A. (1992) *Tetrahedron Lett.* **33**, 5033-5036.

(95) Mullis, K.B. *Scientific Am.* **262**(4), 36-43.(1991)

(96) Mullis, K.B.; Faloona, F.A. (1987) *Methods in Enzymology,* 155, 355-350.

(97) Durand, M.; Chevrie, K.; Chassignol, M.; Thuong, N.T.; Maurizot, J.C. (1990) *Nucleic Acids Res.* **18**, 6353-6359.

(98) Erlich, H.A. (1989): *PCR Technology: principles and applications for DNA applification.* Stockton Press.

(99) Sproat, B.S.; Gait, M.J. (1985) 'Oligonucleotide Synthesis-a practical approach', pp109, IRL Press, Oxford.

(100) Fischer; Phthaler. (1920) *Ber.* **53**, 1606.

(101) Mori, K. (1975) *Tetrahedron,* **31**, 1381-1384.

(102) Darnell, J.; Lodish, H.; Baltimore, D. (1990) '*Molecular Cell Biology*', Scientific American Books.

(103) Catty, D. 'Antibodies-a practical approach.' (1989) IRL Press, Oxford.

(104) Pilz, I.; Schwarz, E.; Palm, W. (1977) *Eur. J. Biochem.* **75**, 195-199.

(105) Horn, T.; Urdea, M.S. (1989) *Nucleic Acids Res.* **17**, 6959-6967.

(106) Chang, C.A.; Horn, T.; Ahle, D.; Urdea, M.S. (1991) *Nucleosides and Nucleotides*, **10**, 389-392.

(107) Horn, T.; Warner, B.D.; Running, J.A.; Downing, K.; Clyne, J.; Urdea, M.S. (1989) *Nucleosides and Nucleotides,* **8**, 875-877.

(108) McCormick, D.B.; Roth, J.A. (1970) *Analytical Biochem.* **34**, 226-236.

(109) McCormick, D.B.; Roth, J.A. (1970) *Methods in Enzymology,* **XVIII**(A), 383-385.

(110) Booth, E.D. (1991) *Ph.D. Thesis*. Edinburgh University.

Chapter Two

Attachment of Lipophilic Molecules to Oligonucleotides during Solid-Phase Synthesis.

A. INTRODUCTION.

10.0. General Principles and Problems of Antisense Therapeutics.

Since the pioneering work of Zamecnik and Stephenson,[1,2] interest in the use of 'antisense' oligodeoxynucleotides (ODNs) as potential therapeutic agents has grown rapidly.[3-8] Disease states which may be treatable with antisense ODNs, include cancer, viral infections, autoimmune diseases, endocrinological diseases, neurological disorders and parasitic or bacterial infections.[7] For a comprehensive review see Uhlmann and Peyman.[9] The basic concepts for the control of gene expression by antisense oligonucleotides are very simple (*Figure 36*).The exogenous antisense ODN is taken up by the cell and hybridises, in a sequence specific manner, to the targeted messenger RNA (mRNA). This prevents the normal process of translation of this particular mRNA into its polypeptide/protein product. The mechanism of translation inhibition (*Figure 37*) may involve prevention of mRNA maturation (splicing, capping or polyadenylation); inhibition of mRNA transport to ribosomes; inhibition of ribosome binding; inhibition of translocation of ribosome along the mRNA; or degradation of the mRNA part of the mRNA-ODN hybrid by RNase H. Inhibition of gene transcription is also possible if the ODN binds to RNA polymerase. However this is likely to be a non-sequence specific process.

Control of gene transcription by 'triple-helix' forming ODNs is another area of great potential for the treatment of cancer and genetic

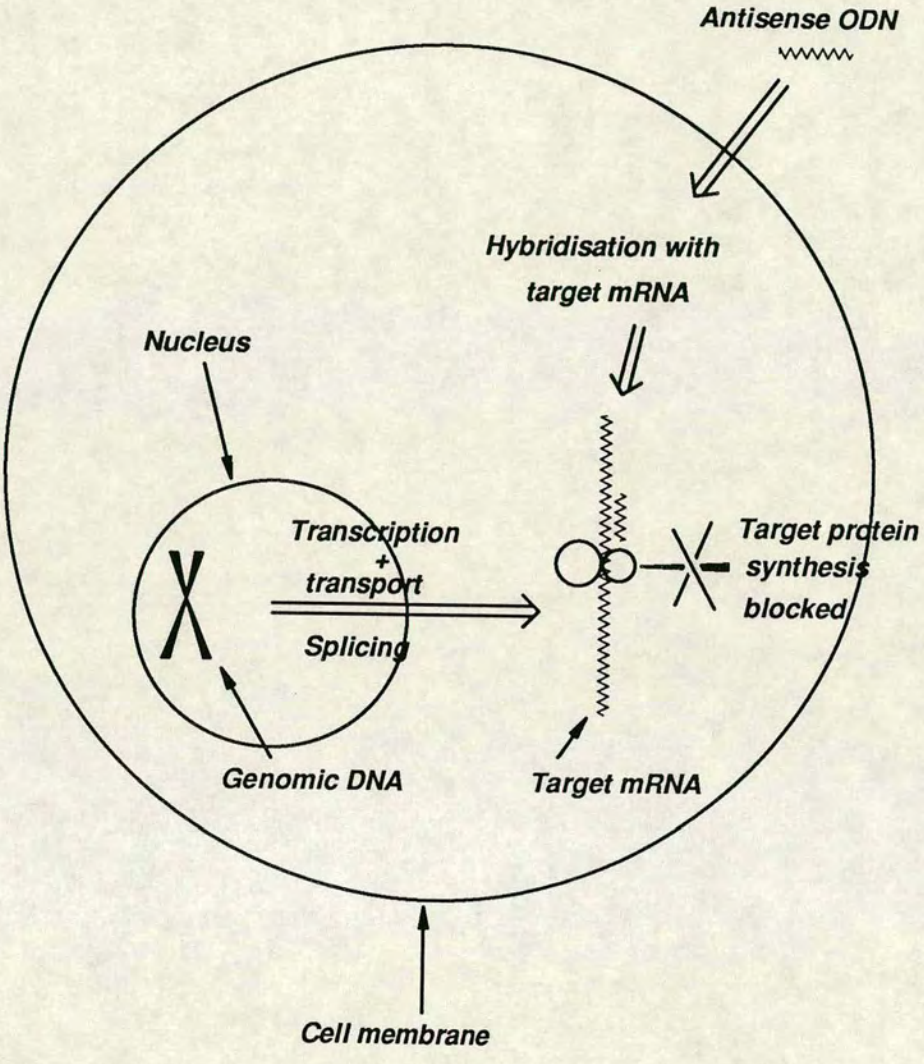
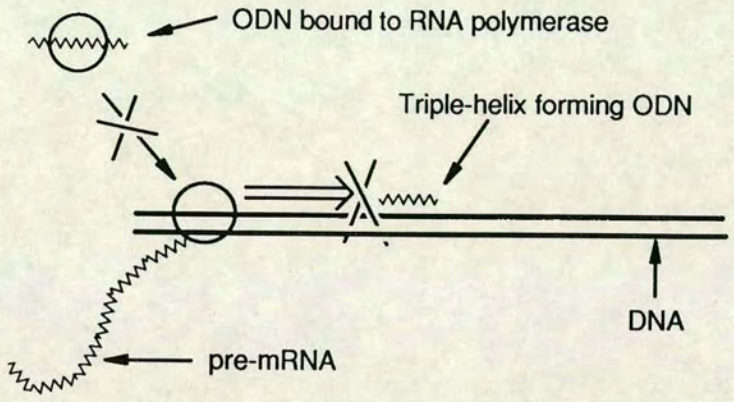
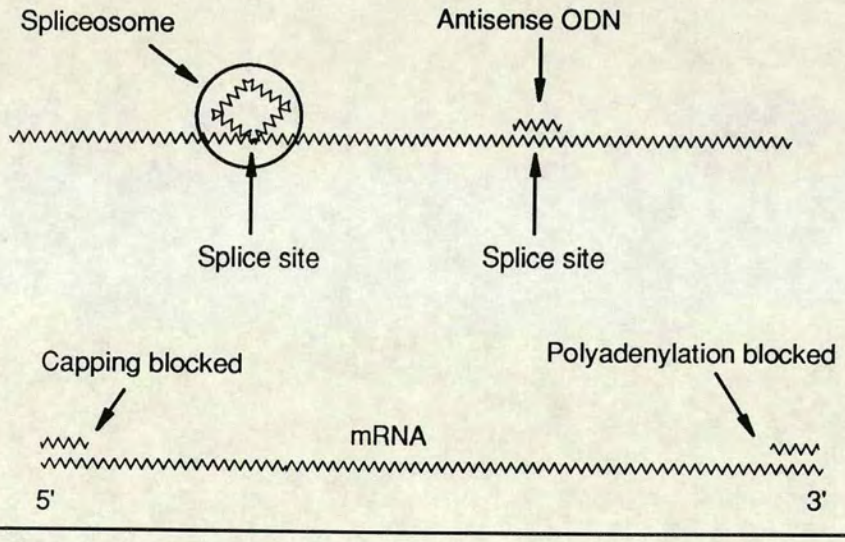


Figure 36: Simple schematic diagram showing the general principle of control of gene expression using antisense ODNs.

(a) Inhibition of transcription



(b) Prevention of mRNA Splicing, capping or polyadenylation.



(c) Translation arrest.

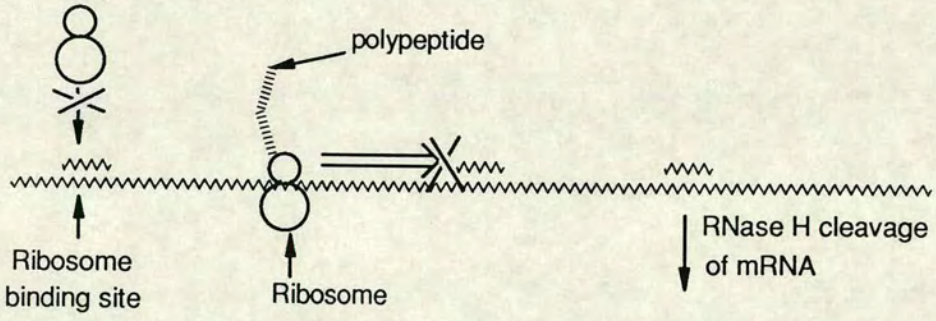


Figure 37: Schematic representation of potential sites of action of antisense ODNs

diseases.[10] This approach involves the binding of an ODN to the double-stranded target sequence in the genome *via* Hoogsteen base-pairing. The resulting triple-helix interferes with the transcription of the target sequence, and thus with the expression of the protein encoded by that sequence.

Although use of antisense ODNs is simple in concept, in practice it is fraught with difficulties. A major obstacle to the commercial feasibility of antisense therapeutics is the high concentration of ODN required to elicit antisense effects. In tissue culture reasonable responses are seen only in the presence of micromolar concentrations of ODN. A major contributor to this low activity is the the poor cellular uptake of ODNs. The work to be described in the Results and Discussion section of this chapter describes attempts to improve the cellular uptake of antisense phosphorothioate oligonucleotides. This introduction will therefore focus on cellular uptake of ODNs and previous attempts to improve it. Other problems inherent in the antisense approach are outlined below. Detailed descriptions of these problems can be found in the review articles already cited.

(i) *Enzymic degradation of oligonucleotides in vivo by endo- and exonucleases.*

This problem has been overcome by the modification of the normal phosphodiester internucleoside linkages to confer resistance to nucleases.[9,11] The two most commonly used modified internucleoside linkages at present are phosphorothioates and methylphosphonates, in which one of the non-bridging oxygen atoms of the normal phosphodiester link is replaced with a sulphur atom or methyl group respectively. These modified backbones can be introduced

conveniently during solid-phase DNA synthesis at any position in an ODN and are largely stable to nuclease activity.

(ii) *Poor water solubility.*

The poor water solubility of non-ionic methyl-phosphonate oligonucleotides (MP-ODNs) is a major drawback of this class of modified ODNs. It can be overcome by the synthesis of ODNs with mixed methyl phosphonate and phosphodiester linkages, however this may compromise nuclease stability.[12] Phosphorothioate oligonucleotides (S-ODNs) are normally readily water soluble due to their ionic nature.

(iii) *Choice and accessibility of the target mRNA.*
(iv) *Non-sequence specific interactions.*
(v) *Cost.*
(vi) *Quality control.*

In spite of the problems encountered in its development, the antisense approach to the treatment of viral and other diseases has made rapid progress. This progress is due in large part to close cooperation between chemists, pharmacologists, clinicians, geneticists, virologists, biochemists, engineers and researchers from many other disciplines. This multidisciplinary approach, coupled with the potential rewards to the pharmaceutical industry of producing effective, non-toxic anti-cancer and anti-viral drugs will continue to fuel progress in the antisense field.

11.0. Cellular Uptake of Antisense Oligonucleotides.

11.1. *Labelling of Oligonucleotides.*

In order to follow the uptake of ODNs into cells the ODNs must be labelled. The two approaches used so far are the attachment of a fluorescent group to the ODN or the incorporation of a radiolabel.

11.1.1. *Fluorescent labelling.*

Labels such as fluorescein[13-15], tetramethylrhodamine, [13,14,16,17] and acridine derivatives [18-21] have been covalently linked to ODNs to allow visualisation and quantitation of uptake. It should be noted that acridine derivatives are also intercalators designed to enhance the duplex stability of the ODN-mRNA hybrid.

Potential complications that arise from the attachment of a fluorescent label to an ODN are:

- The label may be cleaved *in vivo*.
- Large, hydrophobic fluorescent groups may affect the rate or mechanism of ODN uptake.
- The label may affect the intracellular distribution of the ODN.

These complications in the interpretation of results can be partly overcome by the use of appropriate control experiments.

Intracellular distribution can be ascertained directly in living cells by fluorescence microscopy. Quantitation of uptake is possible using Fluorescence Activated Cytometry [15,19,20] or using 'Multiparameter Digitized Video Microscopy.'[16]

11.1.2. *Radiolabelling.*

The enzymic 5'-end labelling of ODNs with ^{32}P commonly used for the detection of oligonucleotide hybridisation probes is inappropriate for *in vivo* applications due to the presence of endogenous phosphatases which cleave off the label. In spite of this obvious drawback many groups have used this method of labelling in uptake studies. Internal ^{32}P labelling[20] can be achieved by ^{32}P end labelling of an ODN followed by template directed ligation with another ODN. ^{35}S labelling of S-ODNs is readily carried out by ODN assembly using H-phosphonate chemistry followed by sulphurization using $^{35}S_8$.[22,23]

Intracellular distribution and quantitation of radiolabelled ODNs is measured by fractionation of cellular components followed by scintillation counting.

11.2. *Uptake of Unmodified and Phosphorothioate Oligonucleotides.*

There is now considerable evidence that normal phosphodiester-linked oligonucleotides (D-ODNs) and S-ODNs are taken-up by cells by receptor mediated endocytosis.[24,25] Loke *et al.* [19] investigated cellular uptake using acridine labelled $(Tp)_n$ of various lengths. Their findings were as follows:

-Uptake of acridine-oligo T was temperature and ATP dependent.

-Uptake of acridine-oligo T was inhibited by unlabelled D-ODN, by S-ODNs and by nucleoside 5'-phosphates (and to a lesser extent nucleoside 3'-phosphates).

-Uptake was not inhibited in the presence of MP-ODNs.

-Uptake was length dependent. Short sequences were taken-up more efficiently.

These results were strongly indicative of the existence of a common cell surface receptor for D-ODNs and S-ODNs. The failure of MP-ODNs to competitively inhibit D- and S-ODNs indicates a different mechanism for the uptake of this class of modified ODN. The putative ODN binding protein was isolated by oligo dT affinity chromatography and was found to have a molecular weight of 80kDa.

Independently, Yakubov *et al.* [26] obtained very similar results using alkylating-ODNs (ODNs linked to a nitrogen mustard moiety). They isolated two possible ODN receptor proteins of molecular weight 79 and 90 kDa. The same group later identified a single 80kDa protein as the putative ODN receptor.[27] The temperature and length dependence of ODN uptake was confirmed by Boiziau and Toulme.[28]

Recently Goodarzi *et al.* [29] reported the existence of a 34kDa membrane protein which had a high affinity for D-ODNs at pH 4-4.5, and a low affinity at physiological pH. This is the reverse of the situation under normal receptor mediated endocytosis conditions where the receptor-ligand complex is dissociated at pH 5 in endosomes.[24] The extreme non-physiological conditions employed may have caused a conformational change in a membrane protein, causing it to bind ODNs.

11.3. *Methyl Phosphonate Oligonucleotides.*

The poor aqueous solubility, and uncharged nature of MP-ODNs has led to the suggestion that they enter cells by passive diffusion.[30] This view has now been challenged.[16,17]

Akhtar *et al.* [17] encapsulated D-, S-, and MP-ODNs in liposomes and measured the rates of efflux by passive diffusion. It was found that MP-ODNs had an efflux half-life of >4 days but the antisense effects of MP-ODNs are observed after a much shorter incubation period with cells.

If liposomes are a reasonable model for the plasma membrane of cells it is unlikely that passive diffusion is the major mechanism of MP-ODN uptake. The water:octanol partition coefficients of MP-ODNs indicated that although they are much more lipophilic than D-, or S-ODNs, they are still much more soluble in an aqueous environment than in the hydrophobic environment that constitutes much of the cell membrane.

Shoji *et al.* [16] found that uptake of a 15mer MP-ODN was temperature dependent, with a sharp rise in uptake occuring between 15 and 20°C. Lowering of cytoplasmic pH, which inhibits receptor-mediated endocytosis, was found to completely prevent uptake of a D-ODN but had no effect on MP-ODN uptake. It was suggested by the authors that MP-ODNs are taken-up by an 'active fluid-phase endocytotic' route, and not by passive diffusion or receptor-mediated endocytosis.

Spiller and Tidd[15] found that uptake of MP-ODNs was not saturable and depended only on the extracellular concentration of the MP-ODN, suggesting passive diffusion. D-ODNs were taken up in a saturable manner consistent with receptor-mediated endocytosis. Surprisingly chimeric ODNs containing only 4 phosphodiester linkages and 10 methylphosphonate linkages were also taken up in a saturable manner. The intracellular concentration of D-ODN and MP-ODN was only 0.8% and 3% of the extracellular concentration, respectively, underlining the extreme inefficiency of cellular uptake of ODNs.[15]

11.4. Intracellular Distribution of Oligonucleotides.

The intracellular distribution of fluorescently labelled ODNs can be conveniently monitored by fluorescence microscopy. Leonetti *et al.* [13,14] used this technique to determine the distribution of microinjected fluorescent α-anomeric,[9] D- and S-ODNs in a variety of cell types. A

rapid accumulation of the ODN in the nucleus was observed. The transport of the ODN to the nucleus was a passive process as it was not temperature or ATP dependent. Nuclear fluorescence persisted for 20 hours for α-anomeric and S-ODNs, but disappeared after 3 hours for D-ODNs, presumably due to nuclease degradation. ODNs containing photoreactive 5-bromo-2'-deoxyuridine were found to crosslink to a variety of proteins, but not histones, on irradiation of the ODN mixed with isolated nuclei. The microinjection of ODNs into the cytoplasm does not accurately reflect the rather more complex process of endocytosis of extracellular ODNs. For example, the packaging of ODNs into endosomes after endocytosis would probably prevent any rapid nuclear accumulation of ODNs.

Using fluorescence microscopy Loke *et al.* [19] observed a punctate pattern of intracellular distribution of acridine-labelled D- and S-ODNs indicative of the packaging of the ODNs in endocytic vesicles. In addition the presence of NaN_3 which inhibits internalization of endocytic vesicles was found to reduce the uptake of radiolabelled ODNs. A punctate intracellular distribution of acridine-labelled D-ODNs was also observed by Saison-Behmoaras *et al.* [21] A radiolabelled ODN was found to have a 2-fold higher incorporation in the nuclear fraction of lysed cells than in the cytoplasm. The localisation of fluorescently-labelled S-ODNs in endosomes or lysosomes was also observed by Bennett *et al.*[23]

Verspieren *et al.* [18] showed that acridine labelled ODNs were evenly distributed throughout trypanosome parasite cells.

In a careful study of the uptake of MP-ODNs and chimeric D-MP-ODNs Spiller and Tidd[15] showed by fluorescence microscopy that fluorescein labelled D-ODNs and D-MP-ODNs were contained within cytoplasmic vesicles, whereas MP-ODNs were evenly distributed

throughout the cytoplasm. In contrast, an equally careful study by Shoji *et al.* [16] found a punctate distribution of fluorescently labelled MP-ODNs. A partial co-distribution of the MP-ODN and FITC-dextran, a marker of endocytic vesicles, was also observed.

11.5. *Summary*

There is considerable variability in the results obtained both for cellular uptake of ODNs and also their intracellular distribution. This is probably a result of a combination of factors including: different cell types; different growth media for cells; the length of the ODN; the presence of different hydrophobic fluorescent labels; the positioning of the fluorophore at the 3'-, or 5'-end of the ODN; and the type of modified backbone used.

However, the following conclusions may be drawn:

(i) Cellular uptake of ODNs is highly inefficient, with intracellular concentrations reaching only a few percent of the extracellular concentration.

(ii) Uptake of MP-ODNs is slightly more efficient than that of D- or S-ODNs.

(iii) D- and S-ODNs are taken up by cells by receptor mediated endocytosis. A putative receptor protein of molecular weight 80kDa has been isolated. The normal function of this protein is not known.

(iv) MP-ODNs are taken up by cells by a non-receptor mediated mechanism. This mechanism is either passive diffusion, or active fluid-phase endocytosis.

(v) Packaging of intracellular ODNs in endocytic vesicles appears to be a common phenomenon. This severely limits the availability of the ODN at its sites of action in the cytoplasm and/or nucleus.

12.0. Efforts to Increase the Cellular Uptake of Antisense Oligonucleotides.

The inefficient cellular uptake of ODNs is a major reason for the limited efficacy of antisense therapeutics. This has led several researchers to devise methods to attempt to increase cellular uptake. The approaches used so far are:

(i) Covalent attachment of a hydrophobic group, such as a hydrocarbon chain, to the ODN to impart general lipophilicity.

(ii) Covalent attachment of groups, such as cholesterol and 1,2-di-O-hexadecyl-3-glycerol, designed to interact with cell membranes or transmembrane transport mechanisms.

(iii) Covalent and non-covalent attachment of peptides and proteins.

(iv) Non-covalent association of the ODN with cationic lipids.

(v) Encapsulation of the ODN in antibody-targeted liposomes.

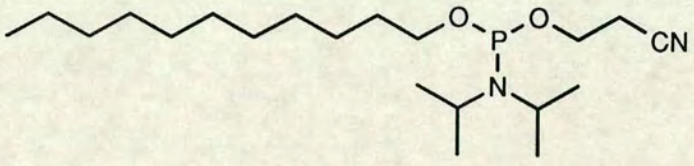
Each of these approaches will be considered individually.

12.1. *Attachment of Hydrophobic Groups to Oligonucleotides.*

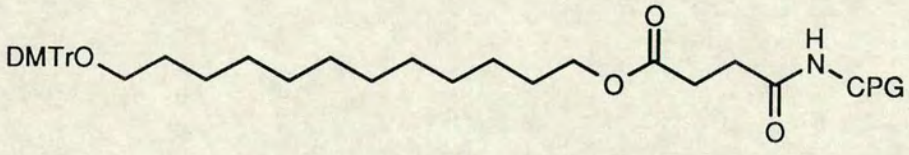
Kabanov *et al.* [31,32] introduced a single undecyl group onto the 5'-end of ODNs using an undecanol phosphoramidite (*Figure 38*), with a yield of 90-95%. The same group was also introduced by carbodiimide coupling to an ODN 5'-phosphate in lower yield. The lipophilic antisense ODNs produced were found to have a considerably greater effect on Influenza virus reproduction and protein synthesis than the corresponding sequence without the undecyl group.

Saison-Behmoaras *et al.* [21] synthesized ODNs with a 3'-dodecyl residue using a dodecane diol solid support (*Figure 38*). It was found that the uptake of a 3'-dodecyl 9mer ODN by human T24 bladder carcinoma

(a) The Undecanol phosphoramidite of Kabanov et al.



(b) The Dodecyl solid-support of Saison-Behmoaras et al.



(c) The 2'-O-nonyl-adenosine phosphoramidite of Guinosso et al.

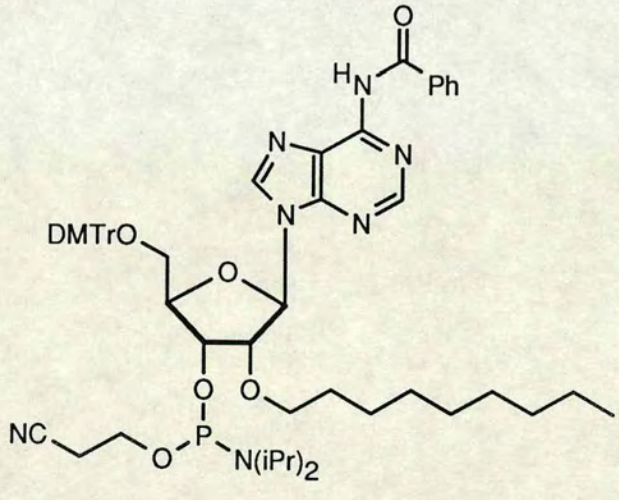


Figure 38: Examples of methods for introducing hydrocarbon chains into ODNs.

cells was 4 times greater than the corresponding unmodified 9 mer but that the distribution of the ODN between the cytoplasm and the nucleus was unaffected. Acridine labelled 3'-dodecyl 9mer ODN was found to be trapped in endosomes, however the distribution of the unmodified acridine 9mer ODN was not reported so no comparison can be made. The dodecyl modified antisense ODNs were considerably more active against *ras* oncogene expression than the corresponding unmodified ODNs.

In contrast Boiziau and Toulme[28] found that the attachment of a dodecyl chain to a D-ODN had only a very marginal effect on uptake.

Abramova *et al.* [33] found that radiolabelled ODNs bearing an octadecyl chain were 'bound to cells' 10 times better than unmodified ODNs and had greater anti-HIV activity.

Guinosso *et al.* [34] synthesized a series of 2'-O-modified *ribo*nucleoside phosphoramidites, including a 2'-O-*n*-nonyl derivative (*Figure 38*). Cellular uptake of ODNs bearing this derivative was not studied, however only modest duplex destabilisation was observed on incorporation of multiple 2'-O-*n*-nonyl adenosine residues into oligonucleotides. In addition, incorporation of 2'-O-modified residues at the 3'-end of ODNs gave significant protection from exonuclease activity.

12.2. *Attachment of Membrane-Interactive Groups to Oligonucleotides.*

12.2.1. *Cholesterol.*

Cholesterol is a major component of cell membranes and is involved in the regulation of membrane fluidity.[24] Cholesterol is transported *in vivo* mainly by lipoproteins, which are found in large quantities in the circulatory system, and are responsible for the transport of hydrophobic molecules. In humans the most important carrier of cholesterol is low-density lipoprotein (LDL).[35,36] LDL is a spherical

particle with a mass of *ca.* 3MDa. It is composed of a core of approximately 1500 molecules of esterified cholesterol enclosed in a coat of approximately 800 molecules of phospholipids and 500 molecules of unesterified cholesterol, and one 400kDa protein called apolipoprotein B-100. The unesterified cholesterol in the LDL coat is in equilibrium with the cholesterol present in cellular membranes.

LDL is taken up by cells by receptor-mediated endocytosis.[24] All cells have high affinity receptors for LDL on their surfaces which are clustered in regions known as coated pits. When LDL binds to its receptor it triggers the polymerisation of clathrin, a protein which is found on the cytoplasmic side of the coated pit. The clathrin polymerisation forms a polyhedral 'basket' which causes the invagination of the membrane of the coated pit, which continues until a clathrin coated vesicle forms. This vesicle, which contains the LDL receptor-ligand complex, then uncoats to form an endosome. The pH in the endosome is then reduced to pH 5, causing the dissociation of the LDL from its receptor. The receptor is then recycled to the cell surface, and the LDL is degraded by enzymes on fusion of the endosome with a lysosome. The time for one complete cycle of endocytosis is *ca.* 10-15 minutes.

This highly efficient mechanism of cholesterol endocytosis has prompted several research groups to attach cholesterol to ODNs in the hope that the cholesterol-oligonucleotide conjugate (Chol-ODN) will associate with LDL and be endocytosed. It should be noted, however, that in most of the literature on Chol-ODNs published so far there is little, or no mention of LDL endocytosis as a potential mechanism of uptake of Chol-ODNs. The enhanced uptake seen in most cases is attributed to the 'membrane anchoring properties' of cholesterol. Endosomal membranes are thought to contain little or no cholesterol[17] which minimises the

potential for entrapment of the Chol-ODN in endosomal membranes by cholesterol anchoring.

Letsinger *et al.* [37] synthesized D- and S-ODNs with a spaced cholesterol attached as a phosphoramidate to the 3'-terminal internucleoside link. This was achieved by oxidative amination of an H-phosphonate linkage using a derivative of cholesteryl chloroformate (*Figure 39*). The attachment of a single cholesteryl moiety did not have a significant effect on the thermodynamic stability of the duplex. The anti-HIV activity (*de novo* infection) of a variety of D- and S-ODNs was considerably enhanced by the attachment of a single cholesteryl moiety. The attachment of a second cholesteryl moiety reduced the activity relative to the mono-cholesteryl ODN. The enhanced activity was also seen for ODNs which contained mismatches to the HIV mRNA. In addition, a 7mer S-ODN with a single cholesterol attached had appreciable anti-HIV activity even though hybridisation to the complementary strand at 37°C was neglible. Clearly a non-sequence specific mechanism of HIV inhibition operates for Chol-ODNs. The outer membrane of HIV (and many other viruses) is rich in cholesterol, with a cholesterol:phospholipid ratio of ~1 (*cf.* 0.5 for most cell membranes).[38] The method used to determine the anti-HIV activity in this study involved the simultaneous addition of the Chol-ODN and the virus particles to cells. This would allow the association of Chol-ODN with the viral membrane, and may interfere with normal interaction of the HIV-membrane glycoprotein gp120 with its CD4 receptor on the cell surface, or with post-binding membrane fusion. This possible mechanism of action is clearly not sequence-dependent.

Stein *et al.* [39] later studied the mechanisms of anti-HIV activity of phosphorothioate oligo dC sequences either with, or without an attached

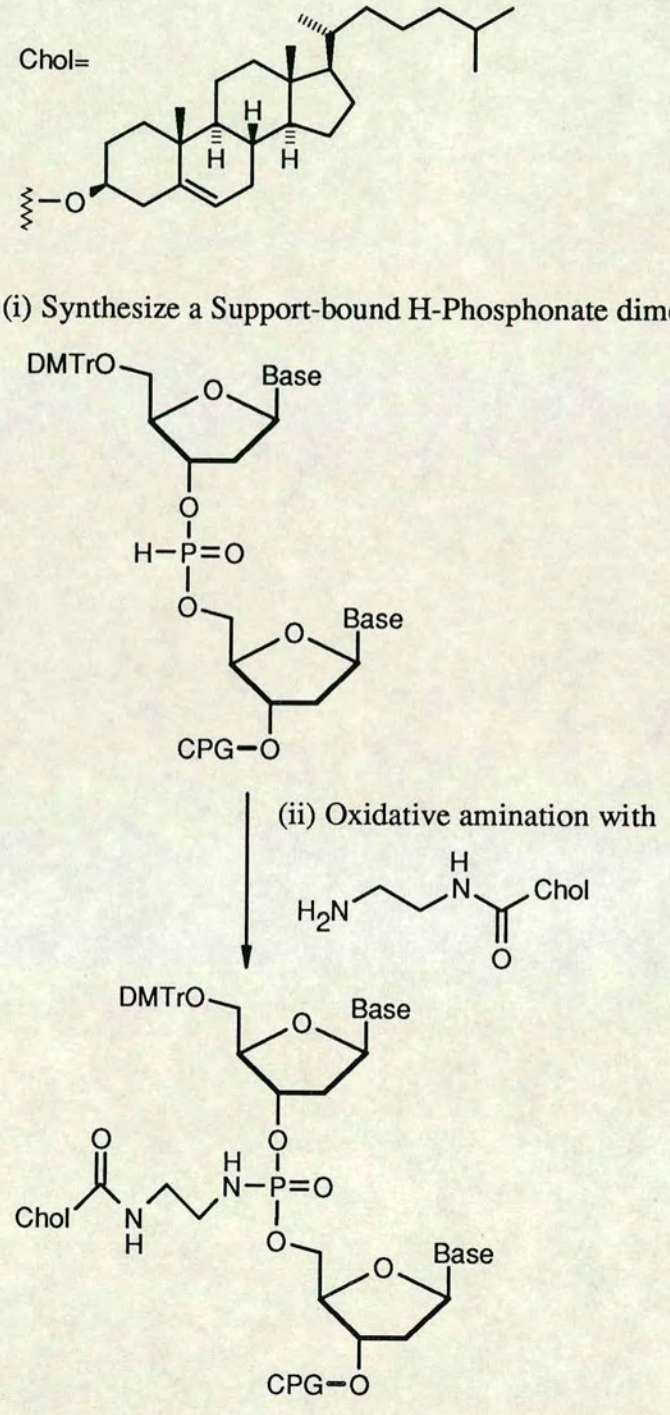


Figure 39: Letsinger's method of synthesizing Cholesteryl ODNs.

cholesteryl group. The cholesterol was attached to the 5'-end of ODNs during solid-phase synthesis using an unspaced cholesterol H-phosphonate monomer. The Chol-S-ODNs had much higher anti-HIV activity than the corresponding unmodified sequences, and were found to inhibit the binding of gp120 to its CD4 receptor and also to inhibit the DNA polymerase activity of HIV1-reverse transcriptase. Both of these inhibitory effects were strongly dependent on the presence of the cholesteryl moiety.

Farooqui *et al.* [40] confirmed the non-sequence specific inhibition of HIV by a variety of spaced Chol-ODNs. Changing the spacer arm from a dimethylene chain to a decamethylene chain had no effect on anti-HIV activity. As well as *de novo* anti-HIV assays, Chol-ODNs were tested on cells which had been incubated with HIV alone for 24 hours. Unlike the *de novo* anti-HIV assays, complete inhibition of HIV protein synthesis (determined by levels of p17 and p24 proteins) could not be achieved, although complete inhibition of syncytia formation was possible. (Syncytia are large, non-viable multinuclear cells caused by fusion of HIV infected cells). A maximum of 80% inhibition of p17 and p24 expression could be achieved under these pre-infection conditions. To determine if the presence of nucleosides in the oligomer was necessary for anti-HIV activity, oligomers were synthesized which were composed of multiple propyl groups linked by phosphorothioate groups and attached to a cholesteryl group. These cholesteryl-anucleosidic phosphorothioates were inactive against HIV, confirming the necessity of the presence of nucleosides for anti-HIV activity.

Ryte *et al.* [27] synthesized radiolabelled D-ODNs with a 5'-alkylating group (a nitrogen mustard) and a 3'-cholesteryl group, and used these in uptake experiments. It was found that most of the Chol-ODNs

were situated in the nucleus or in the cytoplasm (31% and 25% respectively). Surprisingly little was found associated with either the plasma membrane or the with lysosomes (7% and 0.3% respectively). Alkylating D-ODNs were found to associate with, and alkylate an 80kDa protein - the putative ODN receptor. The Chol-ODNs also labelled a 250kDa protein which was not alkylated by unmodified ODNs. It was suggested that this Chol-ODN binding protein may be involved in the endocytosis of this class of modified ODN.

Boutorine *et al.* [41] synthesized radiolabelled alkylating D-ODNs carrying a 3'-terminal cholesteryl group. The uptake of the Chol-ODN was 30-100 times greater than the control ODN. The distribution of the Chol-ODN between the nuclear and cytoplasmic fractions of lysed cells was the same as for the control ODN, with 80-85% in the cytoplasmic fraction and <20% in the nuclear fraction. A more detailed study of the distribution of the ODNs within the cytoplasm and nucleus was not carried out. In another publication, Boutorine *et al.*[42] synthesized a variety of radiolabelled derivatized ODNs, including cholesteryl ODNs. Cystamine derivatized ODNs were reacted with thiocholesterol to give a disulphide-linked 5'-cholesteryl ODN. Alkylation of cystamine derivatized ODNs by iodocholesterol gave a 5'-thioether-linked Chol-ODN. The yields for these processes were 58% and 20% respectively. The labour-intensive procedures and low yields achieved make these methods of linking cholesterol to ODNs feasible only for small-scale research purposes.

Oberhauser and Wagner[43] synthesized 3'-disulphide-linked cholesteryl-2'-O-methyl-RNA oligonucleotides. The cholesterol was attached by reaction of thiocholesterol with a thiol modified oligonucleotide. The ODN was converted to a lipophilic salt to allow it to

be dissolved in organic solvents. The incorporation of cholesteryl oligonucleotides into liposomes was found to be approximately 6 times more efficient than that of their unmodified analogues, opening the possibility of improved encapsulation of ODNs in antibody targetted liposomes (to be discussed in section 12.5. below). The cholesteryl oligonucleotides were found associated with the outside of liposomes as well as in the internal fluid phase, suggesting insertion of the cholesteryl moiety into the lipid bilayer. The binding of cholesteryl oligonucleotides to cells in serum-free medium was 15 times higher than that of the control oligonucleotide and the internalisation was 8 times higher. In the presence of fetal calf serum the rate of uptake of cholesteryl oligonucleotides was reduced, presumably due to association with serum lipoproteins. The intracellular cleavage of the disulphide linkage between the oligonucleotide and the cholesterol was demonstrated. The bioreversible nature of the disulphide linker may increase the availability of the oligonucleotide at its site of action by releasing it from the cholesterol membrane anchor after endocytosis.

De Smidt *et al.* [44] investigated the association of radiolabelled Chol-ODNs with low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Their main findings were:

-Chol-ODNs were completely incorporated into LDL after a 2 hour incubation at 37°C. Under the same conditions there was no incorporation of unmodified ODN.

-The LDL: Chol-ODN ratio in the complex ranged from 1:5 to 1:50.

-When LDL-Chol-ODN complex was incubated with a mixture of LDL, HDL, and albumin, the Chol-ODN was found to be distributed between the LDL and HDL in a ratio proportional to the ratio of the surface areas of LDL and HDL, indicating that the Chol-ODN had no specific affinity

for either LDL or HDL. No Chol-ODN was found associated with albumin. Unmodified ODN did not associate with any protein.

-The plasma half-life for Chol-ODN in rats was 10 times that of a control ODN.

-On injection into rats the Chol-ODN was found mainly in association with plasma HDL (Rat plasma contains predominantly HDL).

-The urinary excretion of Chol-ODNs from rats was found to be lower than that of unmodified ODNs. This reduced excretion is of considerable importance for the potential use of antisense ODNs as therapeutics. The expense of ODNs at present would almost certainly necessitate the recovery of excreted ODN from the urine of patients. Reducing urinary excretion may make this unnecessary.

The methods used for covalently attaching cholesterol to ODNs in the above studies are not readily amenable to routine or large-scale synthesis of Chol-ODNs. Two groups have developed simpler methods of introducing a cholesteryl moiety into ODNs during solid-phase synthesis.

Gmeiner *et al.* [45] synthesised a CPG solid-support based on a cholesteryl 6-aminoethyl *ribo*adenosine derivative (*Figure 40*). This solid-support could be used under normal ODN synthesis conditions to synthesize 3'-Chol-ODNs.

Reed *et al.* [46] synthesized a non-nucleoside-based cholesteryl CPG solid support. The synthetic route is shown in *Figure 41*. Interestingly, Chol-ODNs synthesized by this method had a significantly higher melting temperature (T_m) than the underivatized ODN on hybridisation to a template strand with overhanging ends. This contrasts with the results of Letsinger *et al.* [37] who used a template strand without overhangs. In cells antisense ODNs hybridize with a complementary RNA strand with

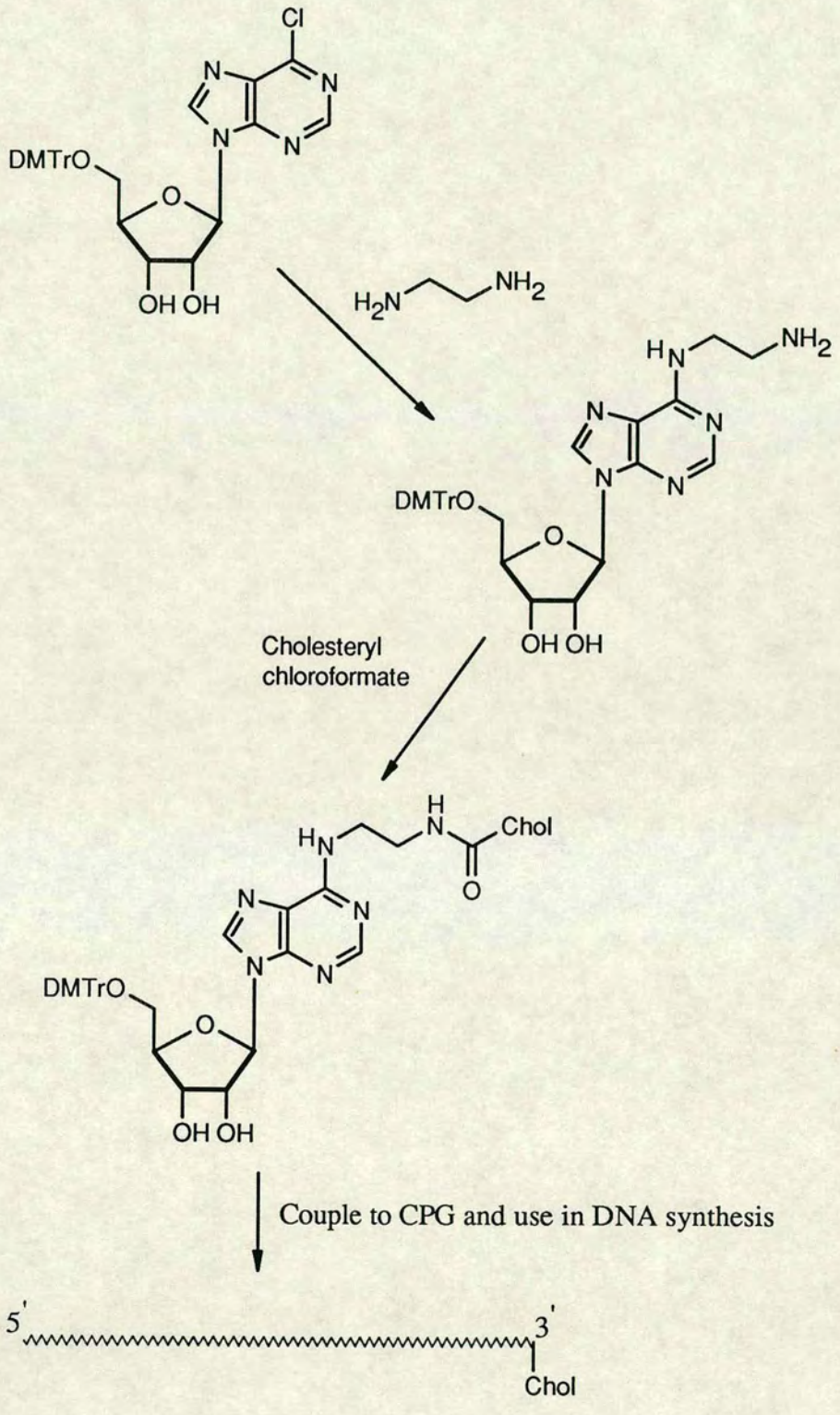


Figure 40: The method of Gmeiner et al. for synthesis of Cholesteryl-ODNs

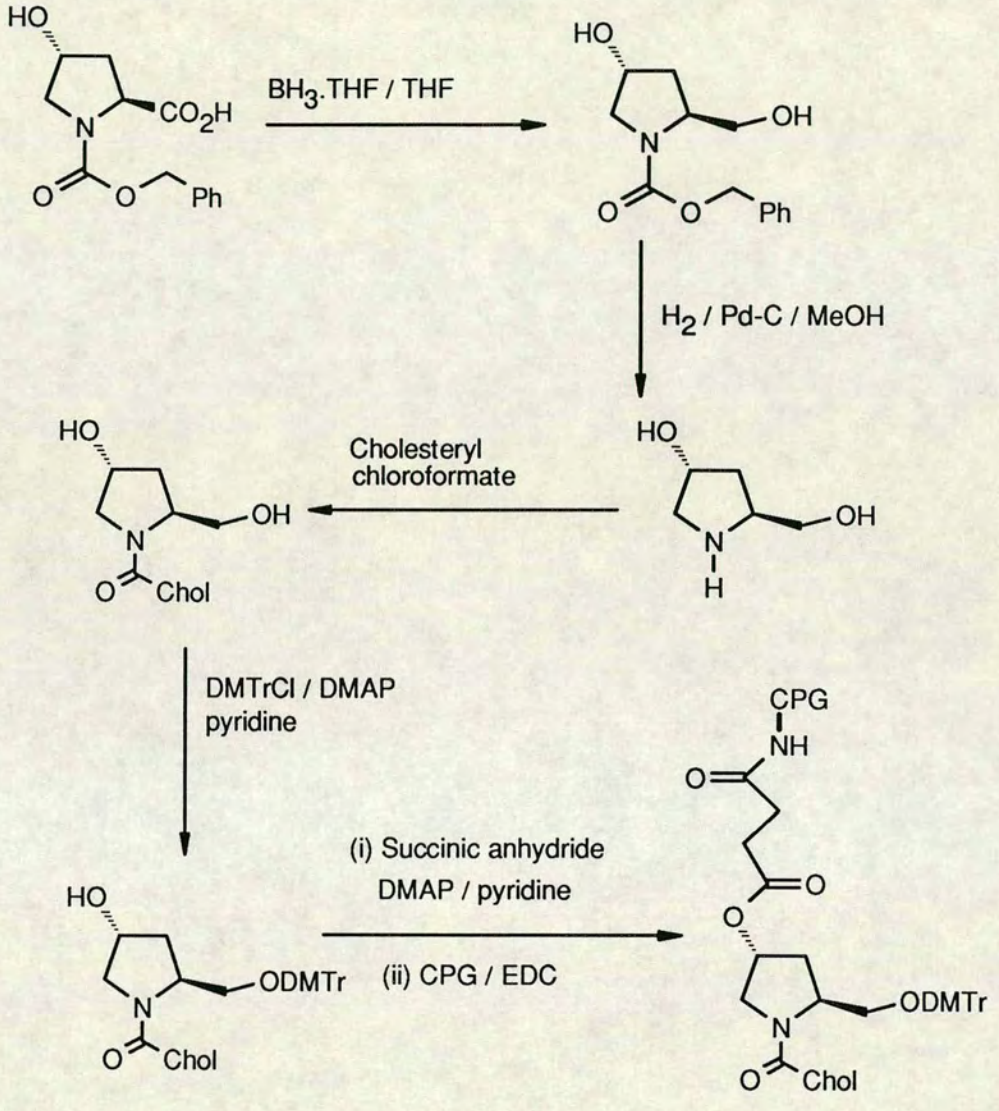


Figure 41: Synthesis of the Cholesteryl solid support of Reed et al.

overhangs of several kilobases. The 'overhanging ends' method of Reed *et al.* [46] is thus a closer approximation to the situation *in vivo*.

12.2.2. *Cholic Acid.*

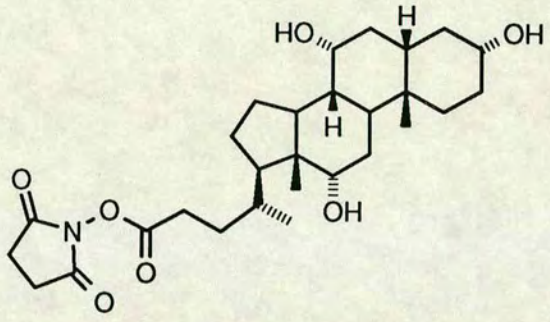
Cholic acid, a metabolite of cholesterol, is a bile salt synthesized in the liver and excreted into the intestines to emulsify fats. It is then reabsorbed and recycled.[24] It has been attached to ODNs as a lipophilic carrier by reaction of its N-hydroxysuccinimide active ester (*Figure 42a*) with an aminomodified ODN. No details were given of the cellular uptake or antisense activity of Cholic acid-ODNs.[47]

12.2.3.*Phospholipids.*

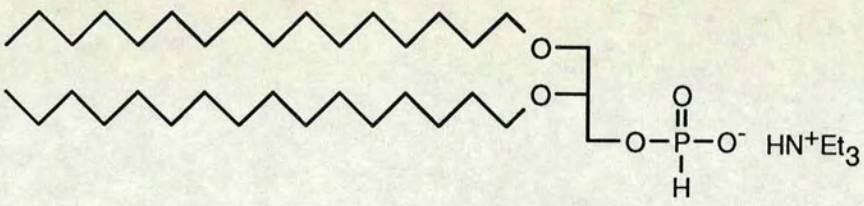
There is one report of the synthesis of phospholipid-oligonucleotide conjugates (PL-ODNs).[48] This was achieved by Shea *et al.* using an H-phosphonate monomer of 1,2-di-O-hexadecyl-*rac*-glycerol during solid-phase synthesis (*Figure 42b*). The T_m of an 11mer PL-ODN was reduced to 34°C compared to 42°C for the unmodified ODN. Uptake studies showed that 8-10 times more PL-ODN was cell associated than the unmodified ODN. *De novo*infection assays against Vesicular Stomatitis virus showed that PL-ODNs were considerably more active than unmodified ODNs or S-ODNs. As with Chol-ODNs the anti-viral effect of PL-ODNs was found to be non-sequence specific.

Dr. Calum MacKellar of this research group synthesized a variety of phosphoramidites to introduce hydrophobic groups into ODNs during solid-phase synthesis. Groups introduced were hydrocarbon chains, a phospholipid (the same as that introduced by Shea *et al.* [48]), and an unspaced cholesteryl group. This work is described in the paper (MacKellar *et al.*) in Appendix 3.

(a) Cholic Acid N-hydroxysuccinimide ester.



(b) The Phospholipid H-phosphonate monomer of Shea et al.



(c) The cationic lipid DOTMA.

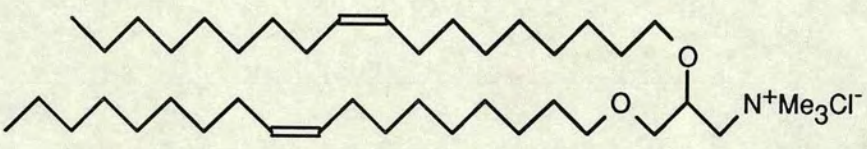


Figure 42

12.3. *Association of Oligonucleotides with Peptides and Proteins.*

The effect of poly(L-lysine) on the cellular uptake of nucleic acids was investigated as long ago as 1974 by Schell.[49] He found that uptake of a variety of single-and double-stranded polyribonucleotides was stimulated either by the formation of ribopolymer-lysine complexes, or by pretreatment of cells with poly(L-Lysine) before addition of the ribopolymer. Association of poly(L-lysine) with the cell membrane gives a net positive charge to the outside of the membrane, resulting in a strong electrostatic attraction for polyanionic nucleic acids.

Lemaitre *et al.* [50] synthesized covalently linked poly(L-lysine)-ODN conjugates (Lys-ODNs). The method used was rather long and involved and certainly not amenable to routine, or scaled-up synthesis. A 3'-ribonucleoside was introduced using pCp and T4 RNA ligase. The terminal phosphate was removed by treatment with bacterial alkaline phosphatase, and the ODN was coupled to poly(L-lysine) by periodate oxidation of the 3'-ribose, followed by reduction with cyanoborohydride. Care had to be taken to prevent precipitation of the conjugate. In spite of these drawbacks, the Lys-ODNs were extremely active (for antisense ODNs) against Vesicular Stomatitis virus protein synthesis, with >95% inhibition at a Lys-ODN concentration of 400nM. This concentration is one or two orders of magnitude lower than for most reported antisense ODNs. Incubation with poly(L-lysine) alone, or with a mixture of poly(L-lysine) and ODN had no effect on viral protein expression, indicating the requirement for covalent linkage of the polypeptide and the ODN. The same group later investigated the antiviral activity of Lys-ODNs which were synthesized by essentially the same method as described above, but using a riboadenosine derivatised solid support to introduce the 3'-terminal ribose moiety.[51]

Stevenson and Iversen[52] found that the cytopathic effects of HIV were considerably reduced by Lys-ODNs at a concentration of 200nM. MP-ODNs were only effective at concentrations of >1μM.

Boiziau and Toulme[28] found that Lys-ODNs led to non-sequence specific inhibition of β-globin synthesis.

Another potential drawback of Lys-ODNs is the cytotoxicity of poly(L-lysine).[9]

Pardridge and Boado[53] found that the uptake of a biotinylated D-ODN was increased from 3% to 15% in the presence of the cationic protein avidin. The anionic protein streptavidin did not have this effect, although it did bind to the biotinylated ODN. Antisense activity was not studied.

Vestweber and Schatz[54] found the covalent attachment of single- or double-stranded ODNs to a mitochondrial precursor protein resulted in the uptake of the ODN into isolated mitochondria. The normal mechanism for the uptake of the peptide appeared to able to cope with the presence of a covalently attached ODN.

Wagner *et al.* [55] investigated the transfection of DNA into cells by association of the DNA with polycation derivatized transferrin. Transferrin is an iron-transport protein which is taken-up by cells by receptor-mediated endocytosis.[24] The ODN-polycation-transferrin complexes were transported *via* the normal transferrin endocytosis pathway, but with lower efficiency than unmodified transferrin.This method of enhancing uptake has not so far been investigated for antisense ODNs.

12.4. *Association of Oligonucleotides with Cationic Lipids.*

The use of cationic lipids to enhance uptake of nucleic acids was first applied to the enhancement of transfection of plasmid DNA into cells.[56,57] This technique is now being applied to enhance cellular uptake of antisense ODNs. This approach is especially attractive as the lipid functionality is not covalently attached to the ODN and may dissociate after endocytosis, thus minimising the possibility of deleterious effects on ODN hybridisation and intracellular distribution. The possible toxic effects of the cationic lipid must also be considered.

Bennett *et al.* [23] found that the potency of an S-ODN-cationic-lipid mixture in reducing the cytokine-induced expression of the membrane protein Intercellular Adhesion Molecule-1 was >1000 times that of the S-ODN alone. The presence of the cationic lipid DOTMA[57] (*Figure 42c*) enhanced the cell association of S-ODN by between 6 and 18 times compared to the control S-ODN alone. The intracellular distribution of fluorescently labelled S-ODN was considerably altered in the presence of the cationic lipid. S-ODN alone was found associated with endosomes or lysosomes. In the presence of cationic lipid the S-ODN was found predominantly in association with the nucleus, with some punctate distribution in the cytoplasm. The nuclear association was seen as soon as 15 minutes after administration of the ODN-lipid mixture, in agreement with the findings of Leonetti *et al.* [13] using microinjected ODNs. The cationic lipid was found to be cytotoxic at concentrations >2μM.

12.5. *Encapsulation of Oligonucleotides in Antibody-Targetted Liposomes.*

Liposomes are attractive candidates as carriers of antisense ODNs. ODNs encapsulated within liposomes are protected from nuclease digestion. The liposomes can be targetted to specific cell types by coupling to antibodies, thus reducing the ODN concentration required for efficacy.

This approach to the delivery of antiviral ODNs has been investigated by Leonetti *et al.* [58] ODNs were encapsulated in liposomes with an efficiency of only 3%. The ODN-liposomes were coupled to Protein A, and these were incubated with cells in the presence of an antibody to a cellular protein. The encapsulated ODN was resistant to nuclease digestion. After 4 hours vesicular stomatitis virus was added. The ODN-liposome inhibited VSV by >95% at an ODN concentration of 800nM. The unencapsulated ODN gave 90% inhibition at 40μM. The viral inhibition was dependent upon the correct antibody being present. The mechanism of uptake was not investigated.

12.6. *Summary and Aims.*

Many of the methods used to increase cellular uptake of antisense ODNs have been successful in increasing the activity of the ODNs. However in some cases the increased activity was non-sequence-specific. This is especially true of ODNs modified with lipophilic groups, such as cholesterol. In spite of this, the potential use of the well-characterised receptor-mediated endocytosis pathway of low-density lipoprotein to transport ODNs into cells remains an attractive option which warrants further investigation. The synthetic routes to ODNs modified with lipophilic groups are at present tedious and not amenable to routine

synthesis of the large quantities of lipophilic ODNs required for biological and clinical testing.

The aim of the work described in this chapter was to synthesize phosphoramidites of lipophilic molecules to allow the routine synthesis of ODNs modified with lipophilic groups.

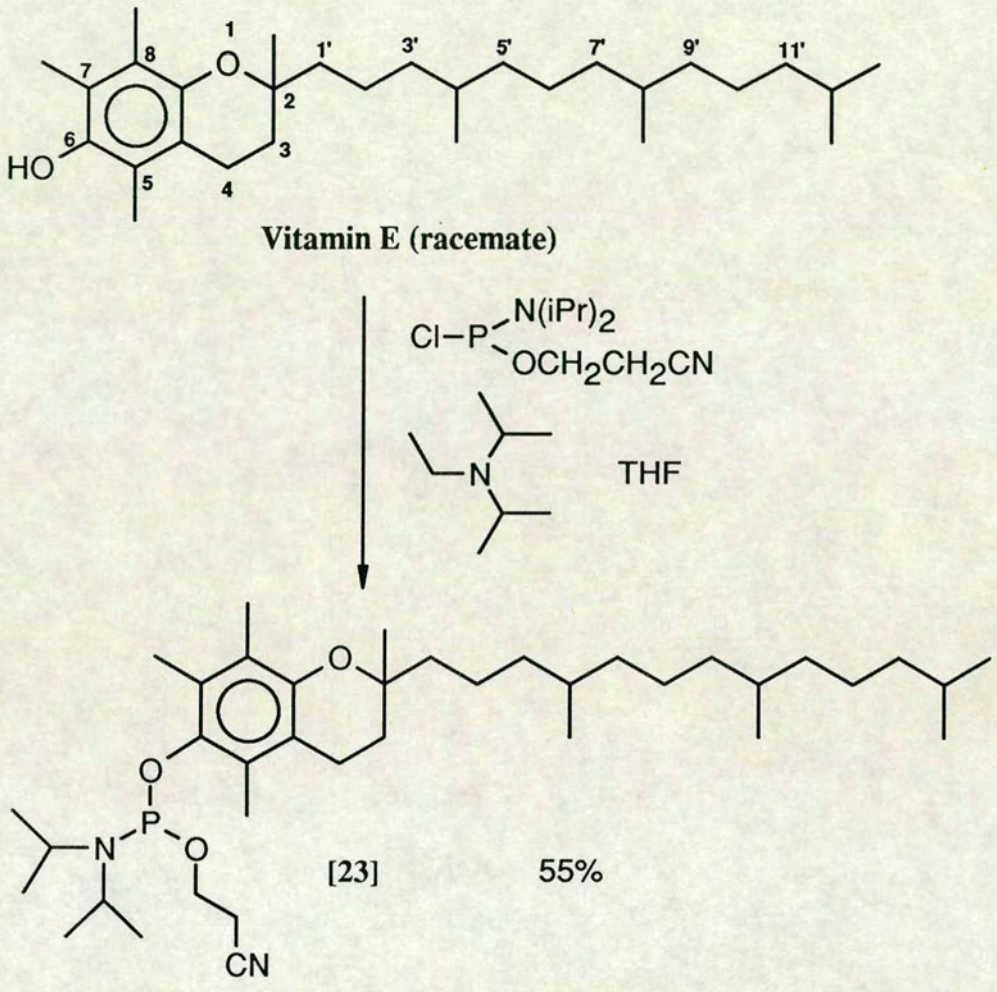
B. RESULTS AND DISCUSSION.

Three types of lipophilic group were attached to ODNs during solid-phase synthesis, Vitamin E, cholesterol and adamantane, using either phosphoramidites of the lipophilic molecules, or a derivatised solid-support.

13.0. Vitamin E.

Vitamin E (α-tocopherol) is an attractive candidate as a lipophilic carrier. It is inexpensive, essentially non-toxic, and has only one functional group for derivatization (*Scheme 7*). It has not previously been attached to ODNs. *In vivo* it is transported in lipoproteins,[59] and in cells it is found mainly in association with the membranes of subcellular organelles[60] such as the endoplasmic reticulum and mitochondria, and when linked to ODN analogues, it may transport them to these sites. The phytyl chain of the Vitamin E buries itself in the membrane and the phenol group protrudes towards the surface.[61,62] Thus attachment of the ODN to the phenolic hydroxyl group should not adversely affect the membrane interaction. Attachment of Vitamin E to ODNs may be particularly useful for the antisense inhibition of *secreted proteins* as these are synthesised on ribosomes attached to the endoplasmic reticulum.

The immobilisation of ODNs derivatised with lipophilic groups on hydrophobic membranes may have other applications in the areas of hybridisation and the capture of PCR products. Samples of the Vitamin E phosphoramidites described below have been sent to Dr. Stephen Will and Dr. Corey Levenson of Roche Molecular Systems Inc. who will investigate this potential application of lipophilic ODNs.



Scheme 7: Synthesis of an unspaced Vitamin E phosphoramidite.

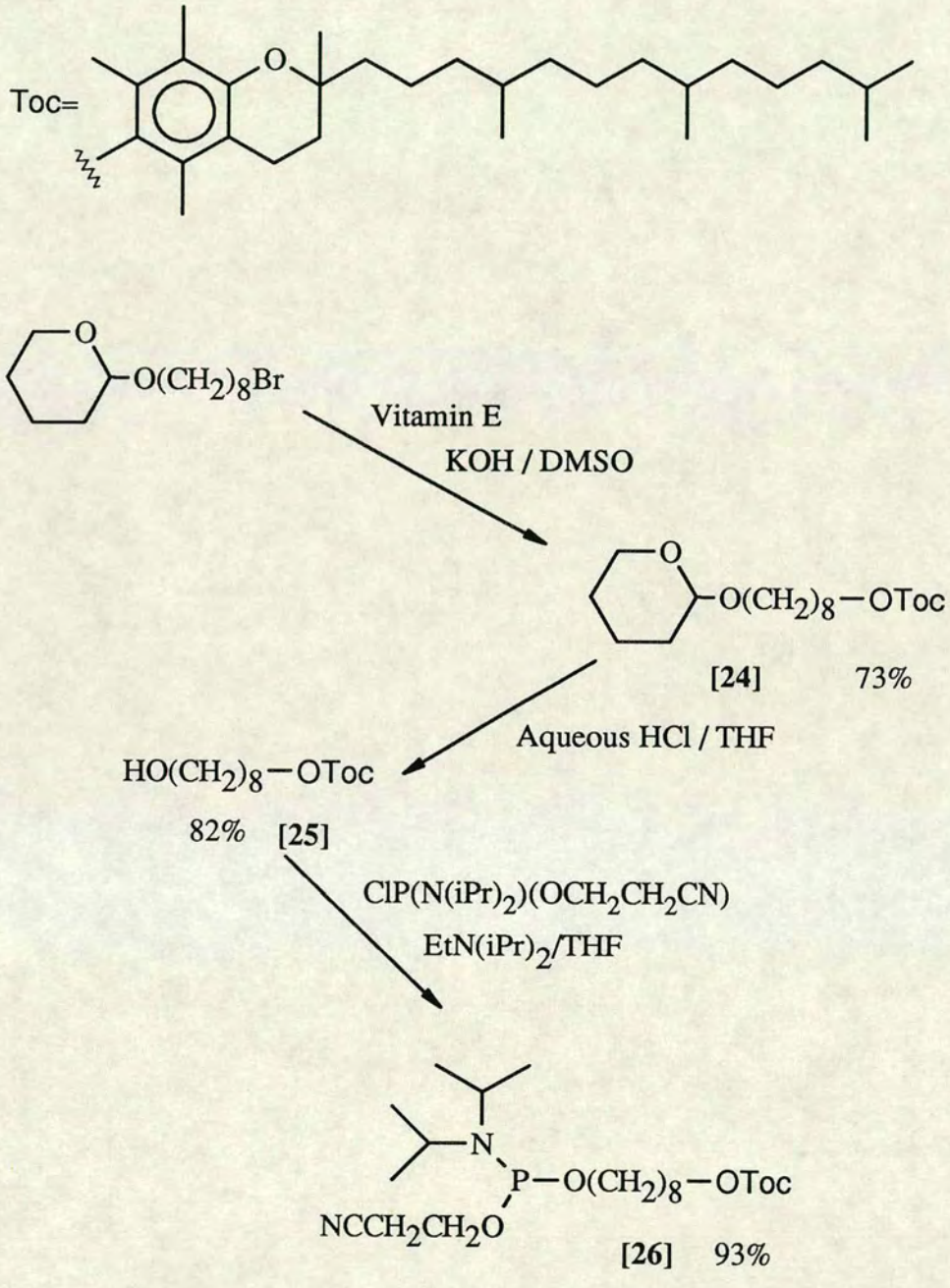
As the properties of ODNs might differ depending on whether the Vitamin E moiety is attached to the 5'- or the 3'-end of the ODN, methods were developed for both.

13.1. *Vitamin E Phosphoramidite Synthesis*

In order to attach Vitamin E derivatives to the 5'-end of ODNs two phosphoramidite monomers were synthesized for use during normal automated solid-phase DNA synthesis.

An unspaced Vitamin E phosphoramidite **[23]** was prepared by phosphitylation of Vitamin E with 2-cyanoethyl N,N-diisopropyl phosphoramidochloridite and diisopropylethylamine in THF (*Scheme 7*). This phosphoramidite is easy to prepare and is ideal for the direct attachment of Vitamin E to ODNs.

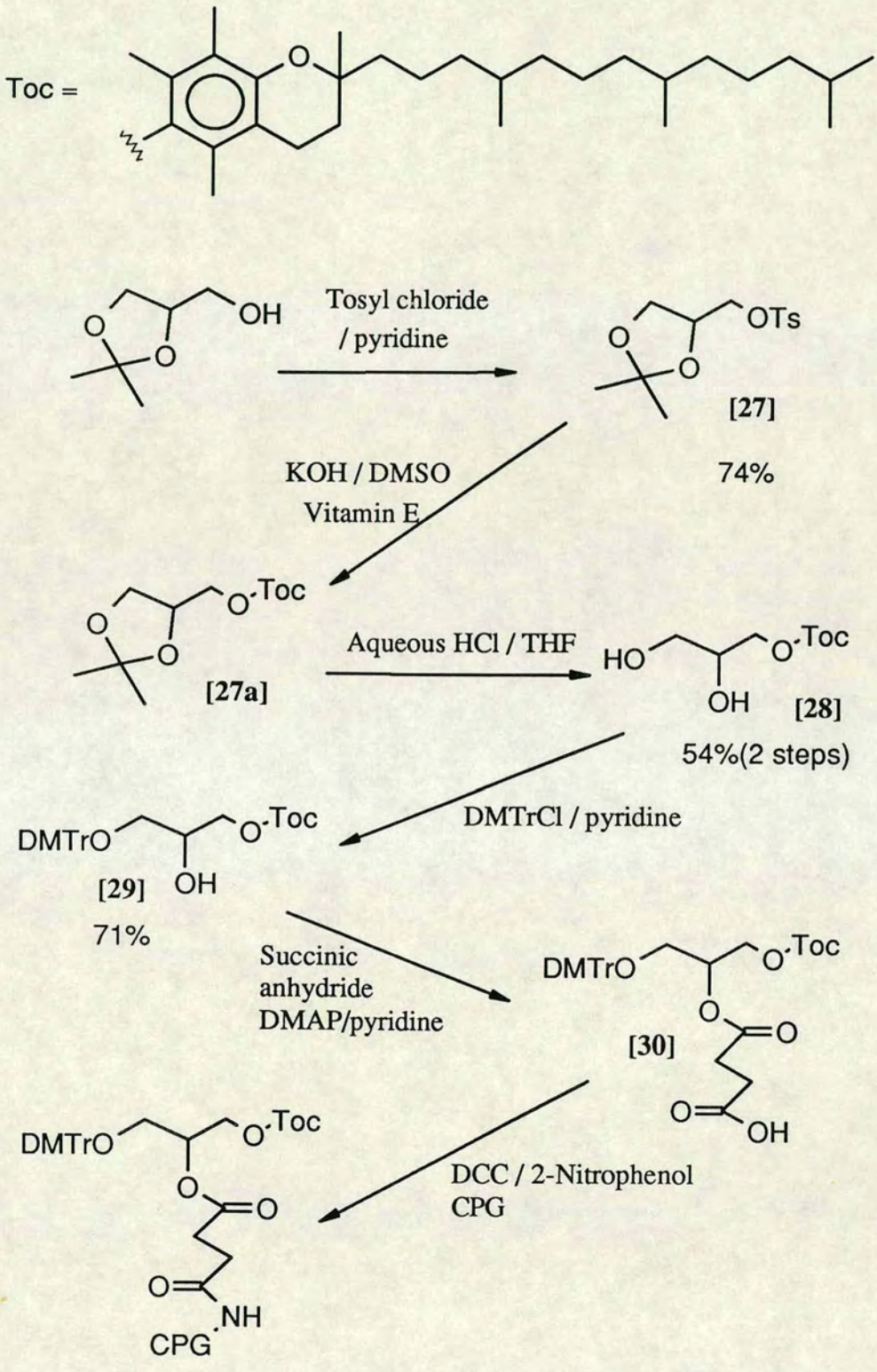
For optimum interaction with membranes and LDL, and to minimise interference with hybridisation it may be necessary to incorporate a spacer between the ODN and Vitamin E. To this end the octamethylene-spaced Vitamin E phosphoramidite **[26]** (*Scheme 8*) was synthesized. Vitamin E was reacted with commercially available 1-Bromo-8-tetrahydropyranyloxy-octane in DMSO in the presence of powdered KOH[63] at room temperature to form the ether **[24]**. This method of ether formation is easy and convenient as it does not require the use of anhydrous solvents and reagents. Both of the starting materials and the product of this reaction ran rather close together on tlc. To ensure an easy chromatographic purification of the product it was essential to use an exact 1:1 ratio of the two starting materials. The THP protecting group was removed by treatment with aqueous HCl in THF and the resulting alcohol **[25]** was phosphitylated in the normal way to give the octamethylene-spaced Vitamin E phosphoramidite **[26]**.



Scheme 8: Synthesis of an octamethylene-spaced Vitamin E phosphoramidite.

13.2. *Synthesis of Vitamin E derivatised CPG*

Attachment of Vitamin E to the 3'-end of ODNs requires a different approach. For this purpose it was necessary to functionalise Vitamin E with two hydroxyl groups, one to link the monomer to the controlled pore glass solid support and the other to permit ODN chain extension (*Scheme 9*). Thus, solketal was reacted with 4-toluenesulphonyl chloride in pyridine and the resulting tosyl solketal **[27]** was reacted with Vitamin E in DMSO in the presence of powdered KOH[63] to form the ether **[27a]**. This compound was passed down a short silica gel chromatography column to remove polar impurities then the isopropylidene protecting group was removed by treatment with aqueous HCl in THF to give the diol **[28]**. Chromatographic purification of this diol was very easy as all the impurities present were very much more lipophilic than the product. The primary alcohol was reacted with dimethoxytrityl chloride in pyridine to give **[29]**. The secondary alcohol was then reacted with succinic anhydride/ DMAP in pyridine and the resulting dimethoxytritylated Vitamin E glyceryl hemisuccinate **[30]** was reacted with 4-nitrophenol and dicyclohexylcarbodi-imide to generate the 4-nitrophenyl active ester. This was coupled to long-chain alkyl amino CPG to give Vitamin E derivatised solid-support with a dimethoxytrityl loading of 24 $\mu mol.g^{-1}$. CPG functionalization by the procedure of Damha *et.al.* [64] resulted in a very low loading of Vitamin E, probably for steric reasons.



Scheme 9: Synthesis of Vitamin E derivatised controlled-pore glass.

13.3. *Vitamin E-Oligonucleotide Synthesis.*

Normal and phosphorothioate[65] Vitamin E derivatised ODNs were synthesized on an Applied Biosystems 380B DNA synthesizer. For 3'-Vitamin E ODNs, standard DNA and phosphorothioate synthesis cycles were used. For the synthesis of 5'-Vitamin E-ODNs, phosphoramidites **[23]** and **[26]** were used as 0.1M solutions in anhydrous dichloromethane, as both compounds are insoluble in acetonitrile. Minor alterations to the synthesis cycles were necessary to accommodate this solvent change. Dichloromethane wash steps were necessary to prevent precipitation of the monomer in the reagent lines and column on mixing with the tetrazole solution. The coupling efficiencies of **[23]** and **[26]** were estimated by comparison of the integrals of the HPLC peaks for a test sequence **VitE**p$(Tp)_4$T and the failure sequence $(Tp)_4$T which would result from a failure of **[23]** or **[26]** to couple. Using this method the coupling efficiencies of **[23]** and **[26]** were estimated to be >95%. In each final coupling cycle, the trityl-ON configuration was used. Base protecting groups were removed by heating to 55°C in concentrated ammonium hydroxide for 5.5 hours. Vitamin E ODNs were found by HPLC to be stable in concentrated aqueous ammonia at 55°C for more than 24hours. The crude ODNs were lyophilized to remove the ammonium hydroxide solution.

13.4. *Oligonucleotide Purification.*

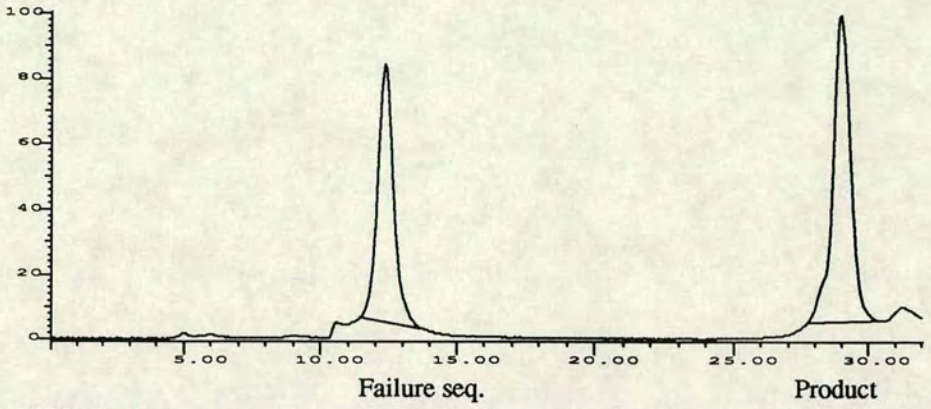
The extreme lipophilicity of VitE ODNs greatly facilitates reversed-phase HPLC purification, the products eluting approximately 15 minutes later than all failure sequences in a gradient of acetonitrile in aqueous ammonium acetate. As the Vitamin E moiety is introduced during solid-phase synthesis with high efficiency, the product is obtained

in high yield. Typical HPLC traces of VitE ODNs are shown in *Figure 43*. The amphiphilic nature of Vitamin E-ODNs makes them behave as surfactants. The use of rotary evaporators for concentrating aqueous solutions of VitE-ODNs is not possible due to extreme foaming of the solution. Lyophilization was found to be a more effective, but more time-consuming, method for concentration of VitE-ODN solutions. Concentration of solutions could also be achieved by passing a stream of argon over the solution, while heating the solution to 40°C in a heating block. After HPLC purification sequences were desalted on NAP-10 columns. Sequences shorter than 12mers were desalted on a larger Sephadex-G10 column.

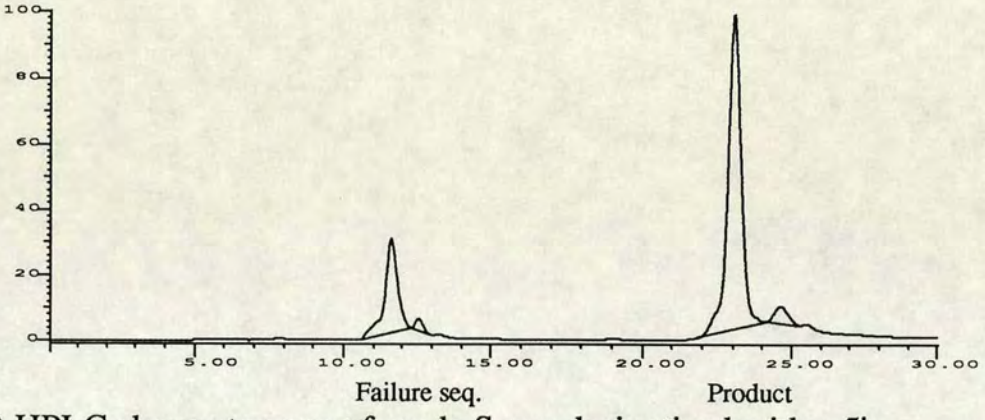
Approximately 10mg (6x1μmol syntheses) of 5'-VitE-p$(Tp)_4$T was synthesized (using **[23]**) and HPLC purified. Analysis by 600MHz ^{1}H-NMR spectroscopy using correlated spectroscopy (COSY) indicated the presence of the ODN and a large number of overlapping signals corresponding to the alkyl protons of the Vitamin E moiety. The ODN signals were assigned using the COSY spectrum. However the overlap in the Vitamin E signals prevented the assignment of individual resonances. The ^{31}P-NMR spectrum was more useful for characterisation of 5'-VitE-p$(Tp)_4$T. *Figure 44* clearly shows the presence of normal phosphodiester signals at around -2 ppm and a signal corresponding to the single aromatic phosphodiester linkage at -4.64 ppm. The ratio of the two signals is 4:1 which is the value expected for this ODN.

13.5. *Synthesis of Anti-HIV Vitamin E-Oligonucleotides.*

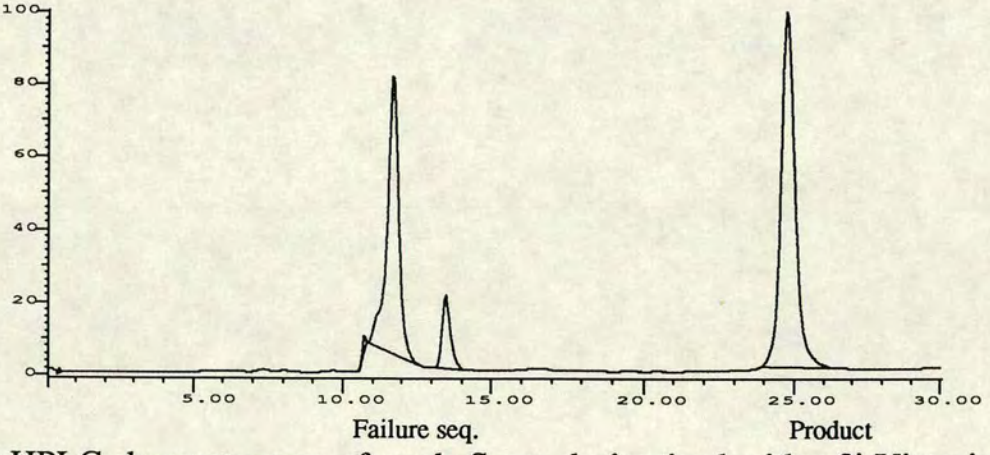
The anti-HIV testing of antisense ODNs in this research group was based on the work of Matsukura *et al.* [66] who found that a phosphorothioate 28mer antisense to a sequence containing the first two



(a) HPLC chromatogram of crude S-*rev* derivatised with a 5'-unspaced Vitamin E using **[23]**.



(b) HPLC chromatogram of crude S-*rev* derivatised with a 5'-octamethylene-spaced Vitamin E using **[4]**.



(c) HPLC chromatogram of crude S-*rev* derivatised with a 3'-Vitamin E using Vitamin E-controlled-pore glass.

Figure 43: Typical HPLC chromatograms of crude 28mer phosphorothioate S-rev derivatised with Vitamin E.

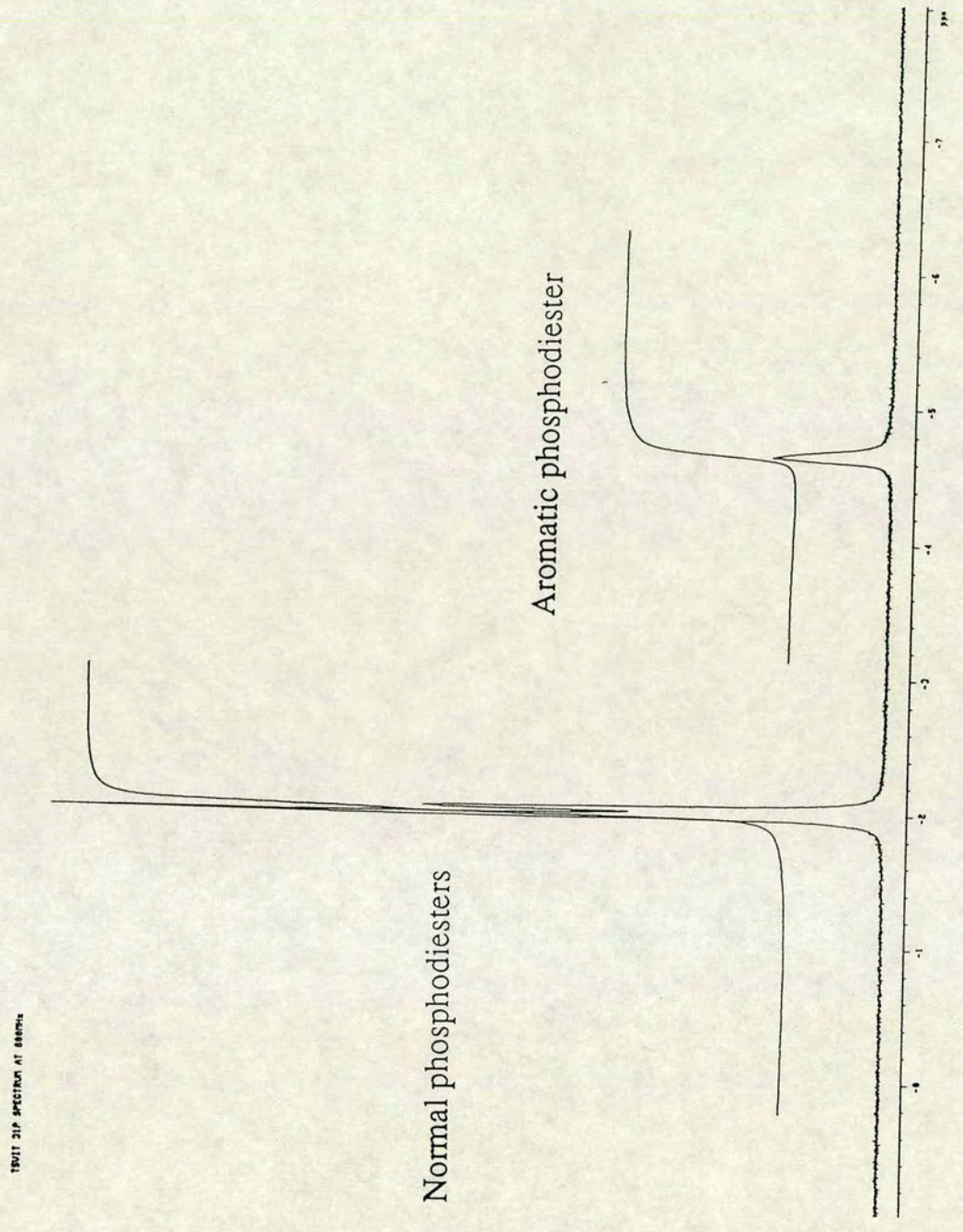


***Figure 44** : ^{31}P-NMR spectrum of unspaced VitEp(Tp)$_4$T in D_2O.*

bases of the start codon of the *rev* gene of HIV-1 (strain IIIB) was effective in inhibiting HIV replication in chronically infected H9 cells. This sequence is:

S-d(TCG TCG CTG TCT CCG CTT CTT CCT GCC A), and will be referred to as S-*rev*.

This test method is likely to give results based on sequence-specific inhibition of HIV replication, rather than non-sequence specific effects which often arise in *de novo* infection assays. Multi-milligram quantities of Vitamin E derivatives of S-*rev* were synthesized with all three types of Vitamin E modification. The VitE sequences have been, or are being evaluated for anti-HIV activity in cell culture.

Three 1.0 μmole scale syntheses of each VitE-ODN were carried out on an Applied Biosystems 380B DNA synthesizer using the 2-cyanoethyl phosphoramidite method. Iodine was replaced with tetraethylthiuram disulphide (TETD) for phosphorothioate synthesis[65] and a 15 minute sulphurization step was used. The Vitamin E phosphoramidite monomers **[23]** and **[26]** described above were used at a concentration of 0.1M in anhydrous dichloromethane. The modified synthesis cycles described above were used. A normal phosphorothioate synthesis cycle was used for the synthesis of 3'-VitE ODNs. The Vitamin E derivatised-S-*rev* sequences were purified by reversed-phase HPLC (injecting one 1μmol synthesis per run), and desalted on NAP-10 columns. Reverse-osmosis purified, UV-irradiated water was used in every stage of purification to ensure the absence of cytotoxic contaminants such as metal ions. After purification the VitE-ODNs were lyophilized and submitted for biological testing. All the VitE-ODNs synthesized were readily soluble in water and the purity of all the lipophilic S-*rev* derivatives was confirmed by denaturing polyacrylamide

gel electrophoresis (7M urea; 20% crosslinking). Visualisation of the ODNs was carried out by UV-shadowing or ethidium bromide staining. Single bands were observed for all the lipophilic S-*rev* derivatives (*results not shown*).

14.0. *Cholesterol.*

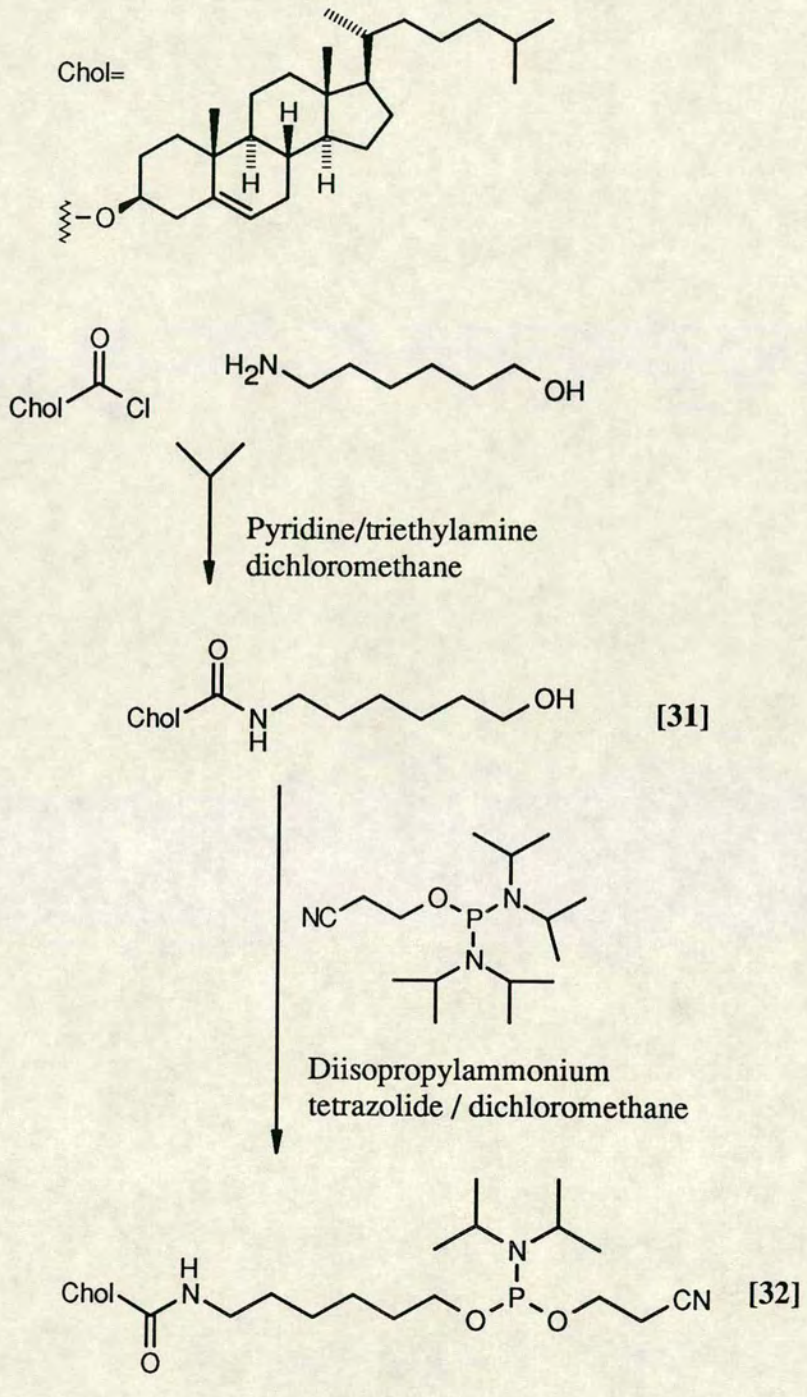
Methods used previously for the attachment of cholesterol to ODNs are discussed in the introduction. In cell membranes, and presumably in lipoproteins, cholesterol is oriented with its hydroxyl function towards the membrane surface and the rest of the molecule buried in the hydrophobic core. The attachment of an ODN to the hydroxyl function should minimise any interference with membrane interactions. A cholesterol phosphoramidite has been synthesized previously by Dr. Calum MacKellar of this research group, by direct phosphitylation of the hydroxyl function of cholesterol. Due to steric hindrance the phosphite intermediate formed during DNA synthesis could not be sulphurised with TETD, and oxidation was only possible on prolonged treatment with iodine. This is clearly an unsatisfactory situation. The steric hindrance problem can be solved by the introduction of a spacer arm between the cholesteryl moiety and the phosphoramidite group. The presence of an alkyl chain attached to the hydroxyl function of cholesterol is unlikely to affect the normal interactions of cholesterol as it is often esterified with fatty acids *in vivo*.

14.1. *Synthesis of a Spaced Cholesterol Phosphoramidite.*

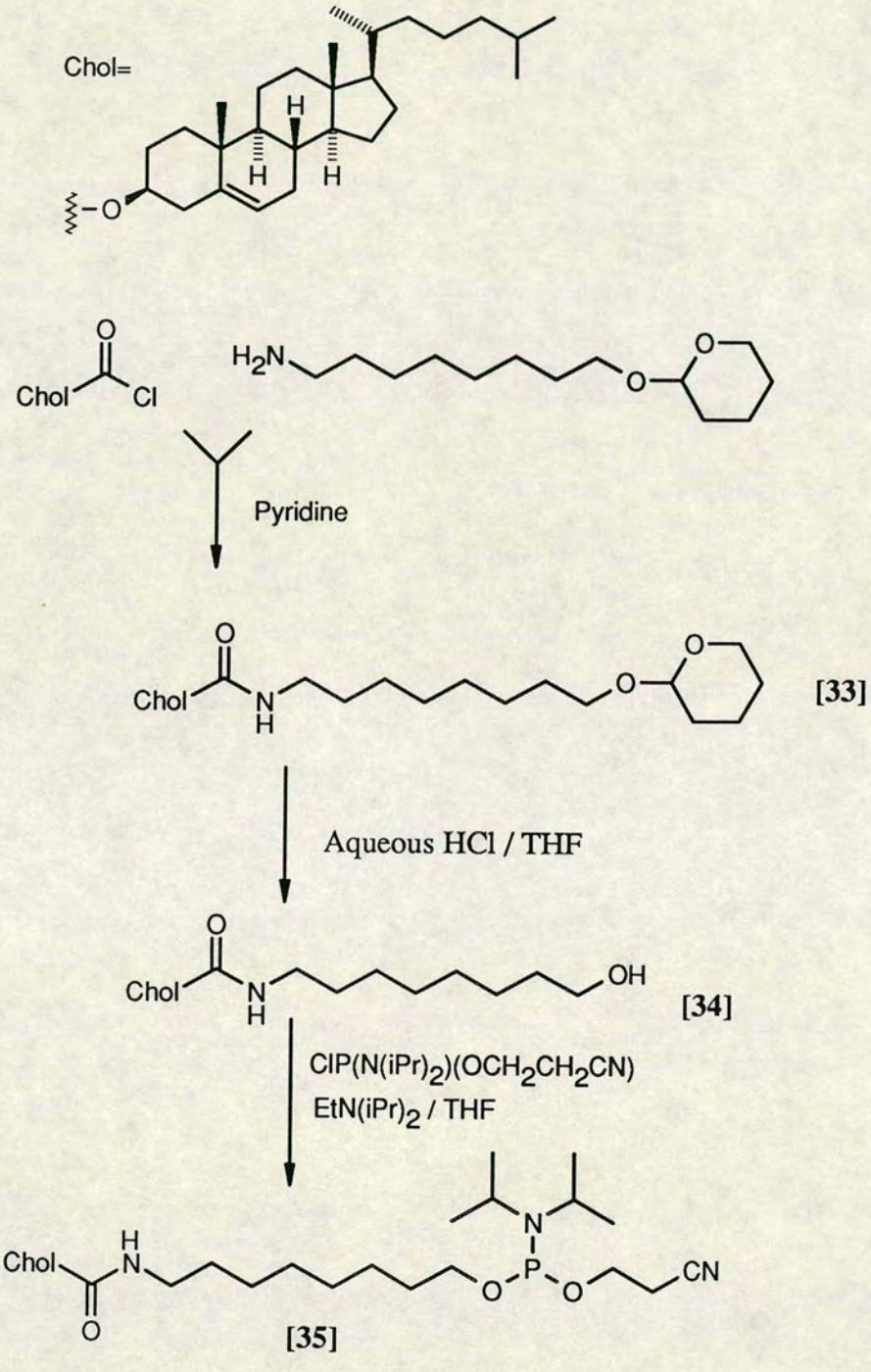
The simplest method of introducing a spacer arm onto cholesterol is by reaction of cholesteryl chloroformate with the amino-function of an amino-alcohol. The carbamate link formed is known to be stable to DNA

synthesis and deprotection conditions.[37,45,46] Thus cholesteryl chloroformate was reacted with 6-amino-hexan-1-ol in pyridine / dichloromethane / triethylamine to give the hexamethylene-spaced cholesteryl alcohol **[31]** (*Scheme 10*). This reaction proceeded in only 47% yield probably due to reaction of the cholesteryl chloroformate with the free hydroxyl functions of both the aminohexanol and the cholesteryl alcohol **[31]**. The hydroxyl function of **[31]** was then phosphitylated using 2-cyanoethyl N,N,N',N'-tetraisopropyl-phosphorodiamidite and diisopropylammonium tetrazolide in dichloromethane to give the hexamethylene spaced cholesteryl phosphoramidite **[32]**.

In order to overcome the problem of side reactions of cholesterol chloroformate at the free hydroxyl functions an octamethylene-spaced cholesteryl phosphoramidite was synthesized using 1-amino-8-tetrahydropyranyloxy-octane **[19]** (*Scheme 11*). The synthesis of this THP-protected amino-alcohol is described in Chapter 1 (section 9.3.). Thus cholesterol chloroformate was reacted with 1-amino-8-tetrahydropyranyloxy-octane **[19]** in pyridine to give the THP-protected cholesteryl octyl alcohol **[33]**. This compound was passed through a short silica gel chromatography column to remove polar impurities, then the THP group was removed using aqueous HCl in tetrahydrofuran. The resulting cholesteryl octyl alcohol **[34]** was obtained in 84% yield. Phosphitylation with 2-cyanoethyl-N,N-diisopropyl-phosphoramidochloridite in the presence of N,N-diisopropylethylamine in tetrahydrofuran gave the octamethylene-spaced cholesteryl phosphoramidite **[35]**.



Scheme 10: Synthesis of a hexamethylene-spaced cholesterol phosphoramidite.



Scheme 11: Synthesis of an octamethylene-spaced cholesterol phosphoramidite.

14.2. *Synthesis and Purification of Cholesteryl Oligonucleotides.*

The synthesis and purification conditions used were essentially the same as those described above for use with the Vitamin E phosphoramidites. The cholesteryl phosphoramidites **[32]** and **[35]** were used as 0.1M solutions in anhydrous dichloromethane. Coupling efficiencies were >95% as estimated by HPLC. As expected the carbamate linkages were stable to DNA deprotection conditions. A typical HPLC chromatogram of a crude cholesteryl ODN is shown in *Figure 45*.

Multi-milligram quantities of cholesteryl derivatised S-*rev* were synthesized, purified and submitted for anti-HIV assays.

15.0. Adamantane.

Adamantane is often used to enhance the lipophilicity of small molecules without introducing an excessive steric bulk. The synthesis of an adamantyl phosphoramidite was easy due to the commercial availability of 1-(1-adamantyl)-ethan-2-ol. This compound was phosphitylated using 2-cyanoethyl-N,N-diisopropylphosphoramido-chloridite in the presence of N,N-diisopropylethylamine in tetrahydrofuran to give the dimethylene spaced adamantyl phosphoramidite **[36]** in 69% yield (*Scheme 12*). This phosphoramidite was used as a 0.1M solution in anhydrous dichloromethane for DNA synthesis using the same conditions as used for the Vitamin E phosphoramidites. Coupling efficiencies were estimated to be >95% by HPLC analysis. As expected HPLC analysis showed that adamantyl ODNs were considerably less lipophilic than the corresponding cholesteryl- or VitE-ODNs (*Figure 46*).

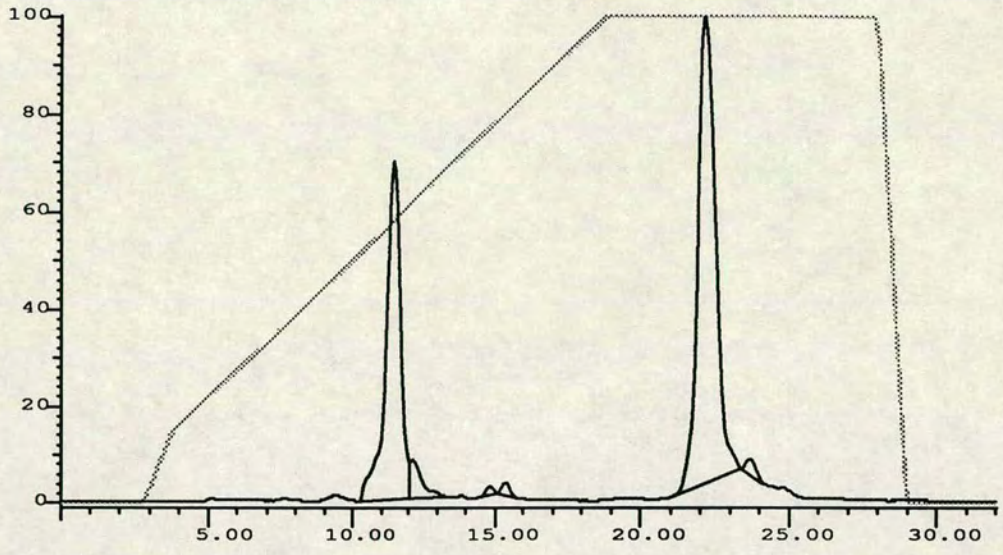
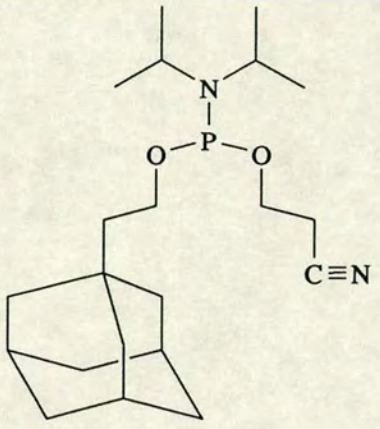


***Figure 45: HPLC chromatogram of crude cholesterol derivatised S-rev synthesized using* [32].**



[36]

Scheme 12: Ethyl-spaced adamantane phosphoramidite.

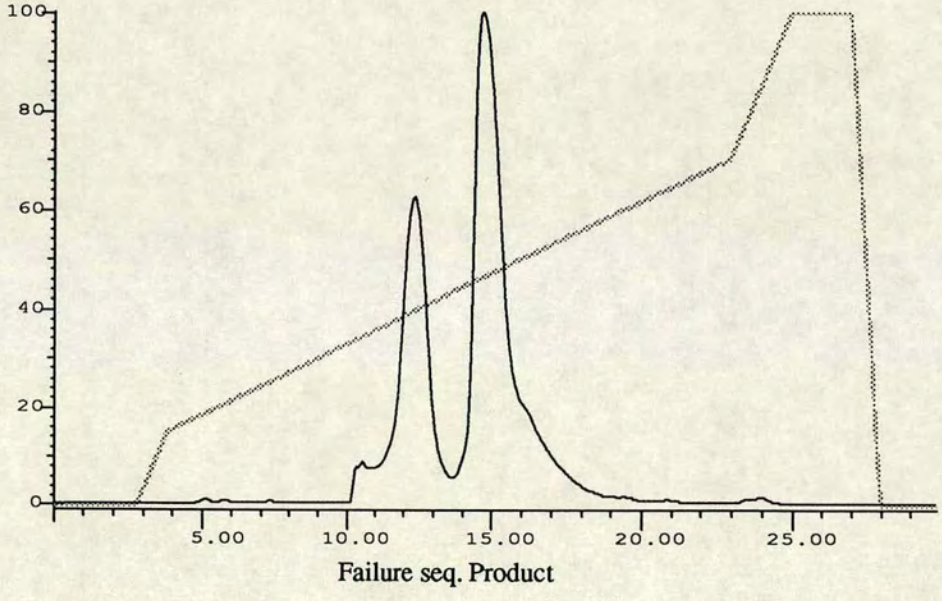


Figure 46: HPLC chromatogram of a preparative injection of crude adamantane derivatised S-rev synthesized using [36].

Multi-milligram quantities of adamantyl derivatised S-*rev* were synthesized, purified and submitted for anti-HIV assays.

16.0. Thermal Denaturation Studies.

In order to determine if the presence of terminal lipophilic groups had an effect on ODN duplex stability, the UV melting temperatures of unmodified and VitE and Chol derivatised S-*rev* with a complementary DNA sequence with four overhanging bases at both the 3'- and 5'-ends were determined. The overhangs, which correspond to part of the *rev* gene, were introduced in order to give a closer approximation to the situation *in vivo* where the ODN will hybridise to a much longer mRNA template. It would have been preferable to use an RNA oligomer complementary to S-*rev*, however the synthesis and purification of a 36mer oligo*ribo*nucleotide is a difficult, time consuming and expensive process. The sequence of the 36mer template ODN was:
d(CCT ATG GCA GGA AGA AGC GGA GAC AGC GAC GAA GAC).
The melting temperatures of the derivatised and underivatised S-*rev* with its 36mer template were measured over a *ca.* 20-fold concentration range and the data obtained were used to determine thermodynamic parameters for each sequence. A single, sharp transition was obtained for all the lipophilic ODNs, indicating no gross interference with hybridisation. A typical UV-melting curve and its first derivative are shown in *Figure 47.* Each curve was determined in triplicate. The concentration dependence of the melting temperature (T_m) confirms the formation of a duplex, rather than a hairpin loop (which melts by unimolecular process), and allows the calculation of thermodynamic parameters for duplex formation.[67-69] For

a heteroduplex the relationship between T_m and concentration is as follows:

$$(1/T_m)= (R/\Delta H^o)\ln(C/4)+(\Delta S^o/\Delta H^o)$$

where ΔH^o=enthalpy of duplex formation,

ΔS^o= entropy of duplex formation,

C= total concentration of single strands.

Thus a plot of $1/T_m$ *versus* ln(C/4) gives a straight line with gradient $(R/\Delta H^o)$ and intercept $(\Delta S^o/\Delta H^o)$.

The free energy of duplex formation ΔG^o can then be calculated using the relationship:

$$\Delta G^o=\Delta H^o-T\Delta S^o$$

Van't Hoff plots of $1/T_m$ *versus* ln(C/4) for S-*rev* and its derivatives are shown in *Figures 49, 49* and *50*. Straight lines were fitted to the data using a linear regression program on a BBC microcomputer. The data used to generate these plots are given in Appendix 2. Thermodynamic parameters and relative T_m values are shown in *Table 1* below.

Sequence	ΔH^o kcalmol^{-1}	ΔS^o kcalmol^{-1}K^{-1}	ΔG^o kcalmol^{-1}	T_m(@1μM) K
S-*rev*	-217.6 (+/-24.4)	-0.611 (+/-0.068)	-35.67 (+/-3.99)	339.64
VitE-S-*rev*	-183.4 (+/-17.0)	-0.508 (+/-0.047)	-32.03 (+/-2.98)	340.79
VitEoct-S-*rev*	-220.6 (+/-16.4)	-0.616 (+/-0.046)	-36.97 (+/- 2.75)	341.30
3'-VitE-S-*rev*	-197.3 (+/-23.9)	-0.549 (+/-0.066)	-33.85 (+/-4.09)	340.81
Chol-S-*rev*	-227.6 (+/-20.4)	-0.639 (+/-0.057)	-37.26 (+/-3.35)	340.26

Table 1 : Thermodynamic parameters of hybridisation of S-*rev* and derivatives with a complementary 36mer template. (Numbers in parentheses are errors).

The minor differences in the thermodynamic parameters and T_m values are within experimental error and it is clear that terminal lipophilic modifications make very little difference to the duplex stability of S-*rev* with its overhanging template strand. Concentration dependent thermal denaturation studies were not carried out on adamantyl derivatised S-*rev*. However at a concentration of 5.5μM adamantyl derivatised S-*rev* hybridised to the 36mer template has a T_m of 342.2K.

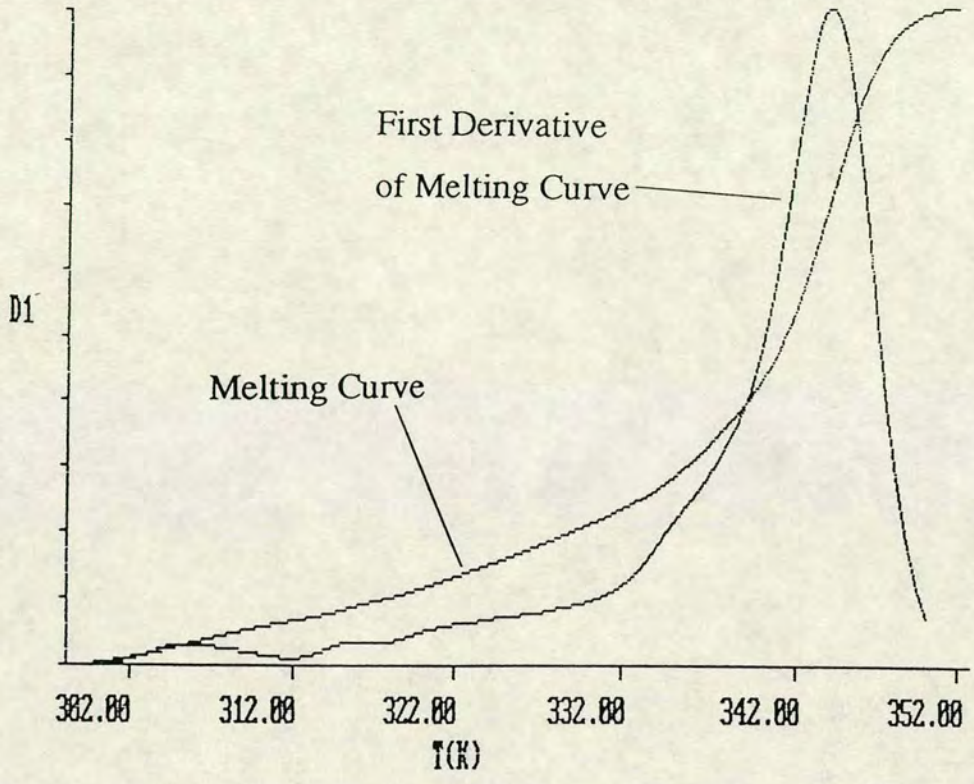


Figure 47: UV thermal denaturation curve and first derivative for VitE-S-rev hybridising to a 36mer template. (Y-axis-arbitrary units).

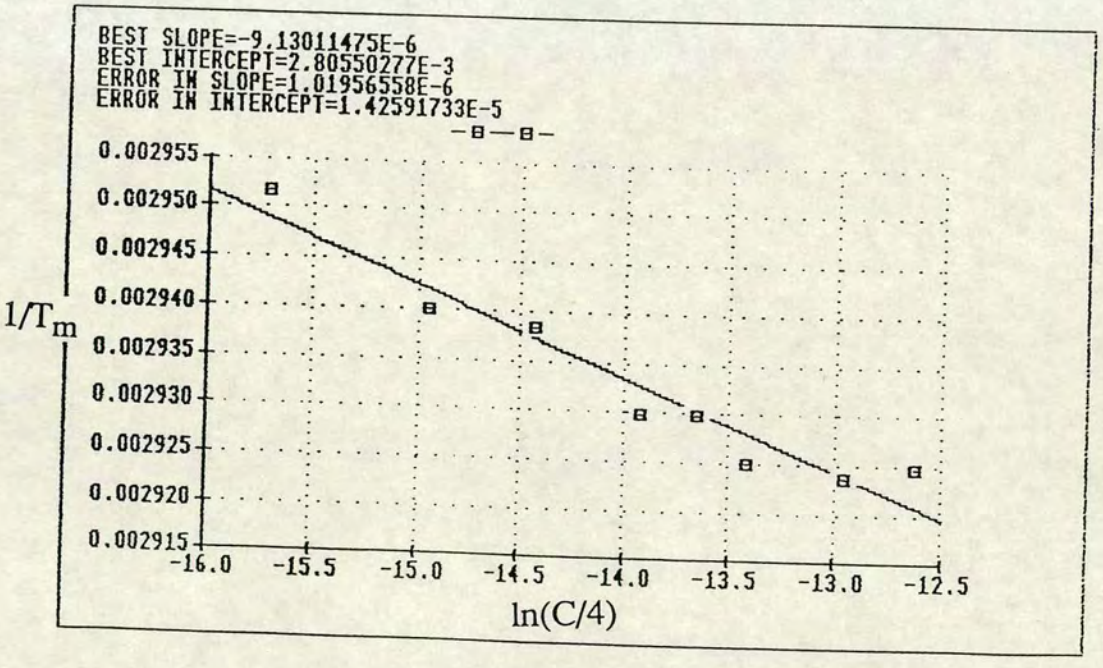
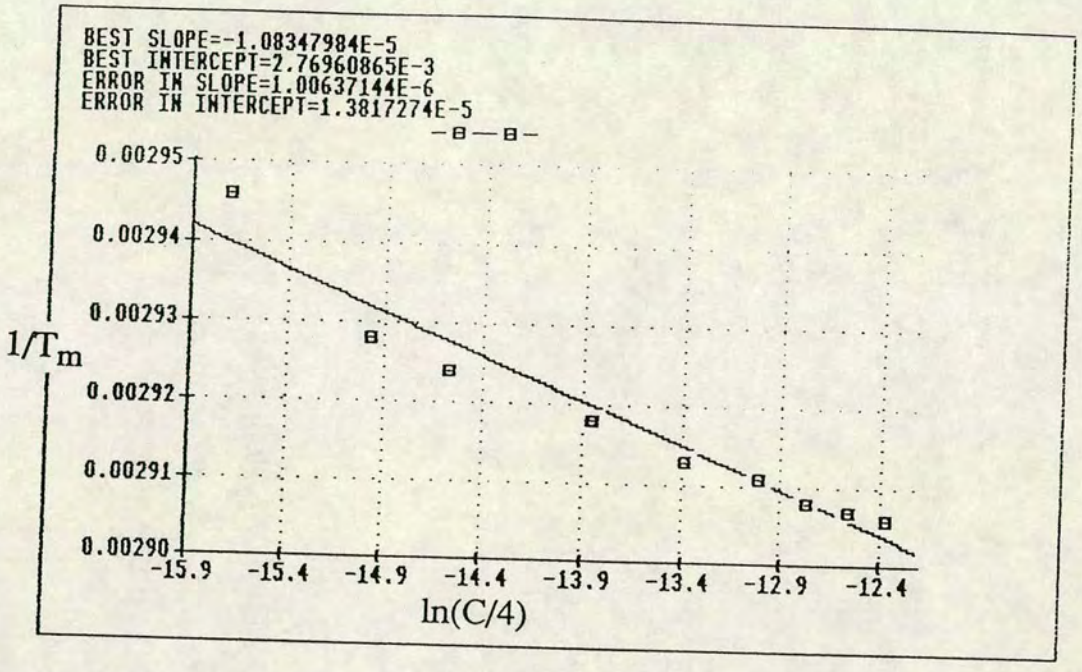


Figure 48: Van't Hoff plot for unmodified S-rev.

(a) Unspaced VitE-S-*rev*.



(b) Octamethylene-spaced VitE-S-*rev*.

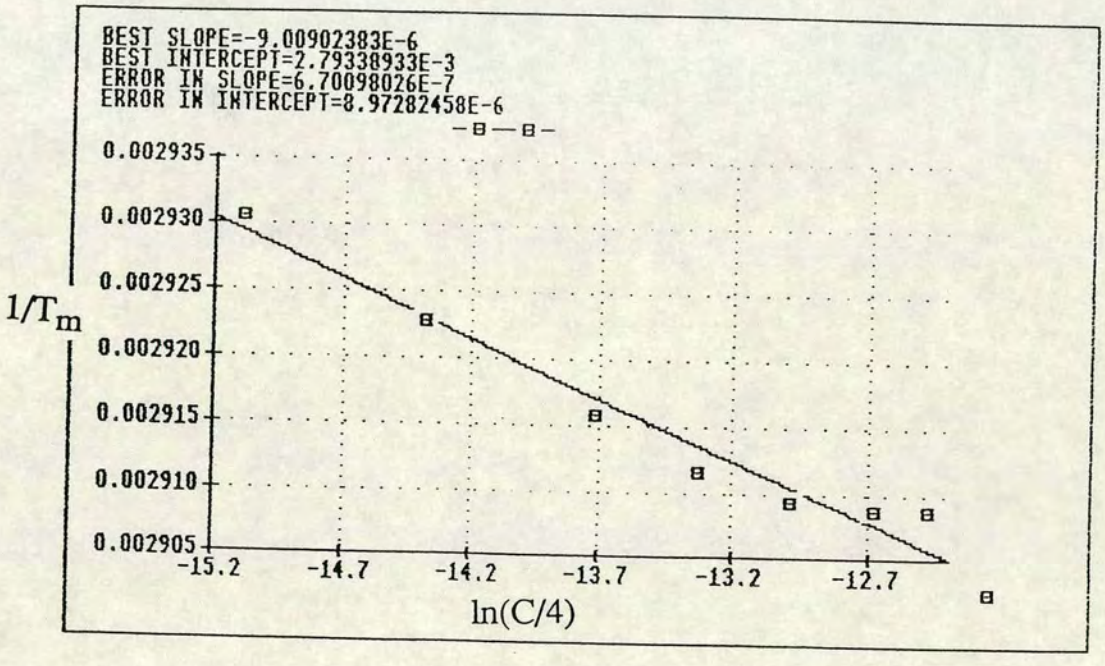
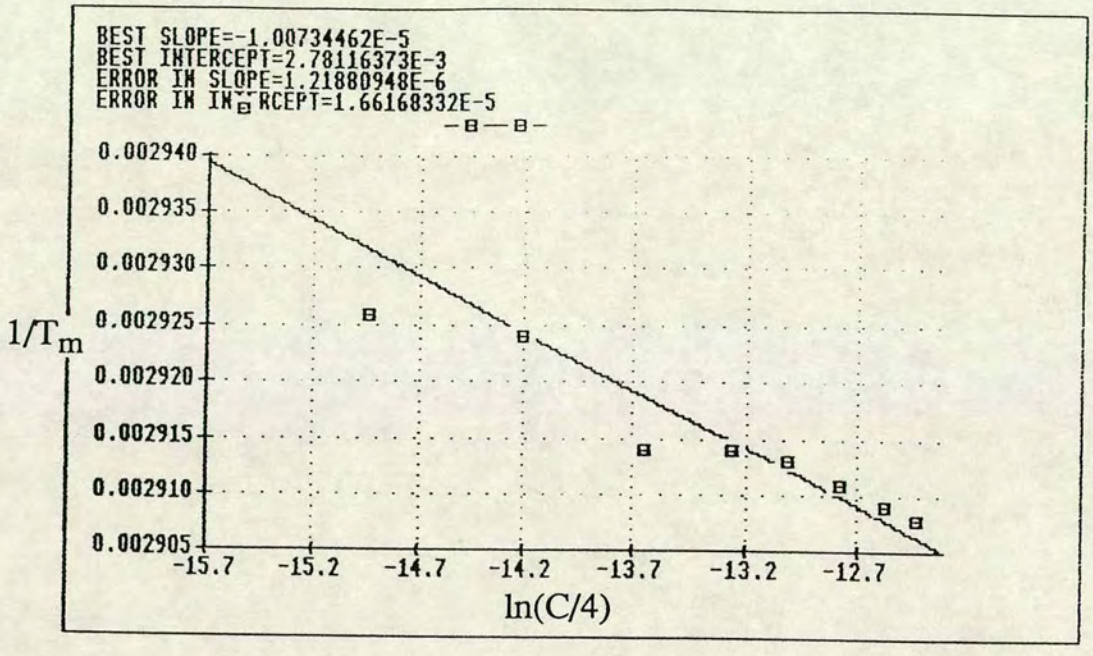


Figure 49: Van't Hoff plots for S-rev derivatives.

(a) 3'-VitE-S-*rev*.



(b) Chol-S-*rev*.

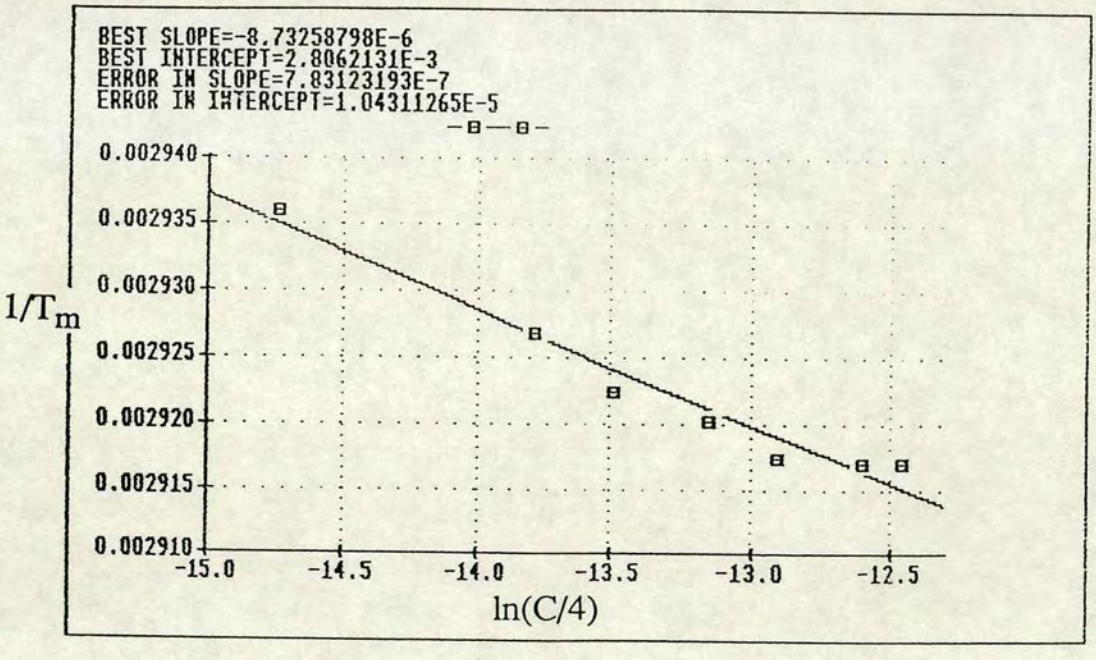


Figure 50: Van't Hoff plots for S-rev derivatives.

17.0. Summary.

(i) Spaced- and unspaced-Vitamin E phosphoramidites have been synthesized in reasonable yields from inexpensive starting materials.

(ii) A Vitamin E derivatised controlled pore glass (CPG) solid-support has been synthesized with a loading of 24 $\mu mol.g^{-1}$.

(iii) Hexa- and octa-methylene spaced cholesterol phosphoramidites have been synthesized in reasonable yields from inexpensive starting materials.

(iv) An ethyl spaced adamantane phosphoramidite has been synthesized.

(v) Conditions have been determined to allow the attachment of Vitamin E, cholesterol and adamantane to ODNs during solid-phase synthesis using the phosphoramidites and Vitamin E CPG.

(vi) Vitamin E, cholesterol and adamantane have been attached to both phosphodiester and phosphorothioate ODNs in high yields (>95%) with minimal changes to standard DNA synthesis conditions.

(vii) HPLC purification of lipophilic ODNs is facile due to the increased lipophilicity of the desired product relative to underivatised failure sequences.

(viii) The lipophilic ODNs synthesized were readily soluble in water.

(ix) Samples of the Vitamin E phosphoramidites have been sent to Roche Molecular Systems Inc. to investigate the possibility of immobilisation of ODNs on lipophilic solid-supports.

(x) Multi-milligram quantities of an anti-HIV 28mer phosphorothioate ODN (S-*rev*) modified with Vitamin E, cholesterol and adamantane have been synthesized, purified and submitted for testing.

(xi) All lipophilic S-*rev* derivatives ran as single bands on denaturing polyacrylamide gels.

(xii) Thermal denaturation studies indicate that there is little significant effect on the hybridisation properties of ODNs on cholesteryl or Vitamin E derivatisation.

(xiii) Vitamin E has not previously been attached to ODNs. The Vitamin E work described above has been published. (Will, D.W.; Brown, T. (1992)*Tetrahedron Lett.* **33**, 2729-2732.)

(xiv) The work carried out on Cholesterol has been published. (MacKellar, C.; Graham, D.; Will, D.W.; Burgess, S.; Brown, T. (1992) *Nucleic Acids Res.* **20**, 3411-3417.)

(xv) Cambridge Research Biochemicals are carrying out trials on the octamethylene-spaced cholesterol phosphoramidite with a view to marketing it.

18.0. Suggestions for Future Work.

(i) Synthesis of a variety of sequences derivatised with lipophilic groups to determine the sequence specificity of any antiviral activity.

(ii) Rigorous studies on both cellular uptake and intracellular distribution of lipophilic ODNs are urgently required. Work carried out in collaboration with John Grzybowski demonstrates that fluorescently labelled VitE-ODNs can be synthesized (*results not shown*).

(iii) The water/n-octanol partition coefficients of lipophilic ODNs should be measured. The possibility of solubilising lipophilic ODNs in organic solvents, such as tetrahydrofuran, would open many interesting avenues of research.

(iv) Lipophilic methylphosphonate ODNs should be synthesized to compare their antiviral and solubility properties with those of lipophilic phosphorothioates.

(v) A practical cholesteryl derivatised solid-support should be synthesized. Dr. Calum MacKellar of this research group synthesized such a derivatized CPG previously, but oxidation and sulphurisation of the first internucleoside linkage was highly sterically hindered. The synthesis of a spaced cholesteryl CPG should be facile.

(vi) The attachment of multiple lipophilic groups to ODNs during solid-phase synthesis should be possible using the lipophilic phosphoramidites in combination with the multiple hydroxyl phosphoramidite **[14]** described in Chapter 1 (section 8).

C. EXPERIMENTAL.

Characterisation of compounds as previously described in Chapter 1 of this thesis.

Thin Layer Chromatography.

Thin layer chromatography (tlc) was carried out on aluminium sheets, silica 60 F_{254}, 0.2mm layer (Merck) using the following solvent systems:

(A) Hexane-ethyl acetate (80:20, v/v)

(B) Hexane-ethyl acetate (90:10, v/v)

(C) Hexane-ethyl acetate (50:50, v/v)

(D) Toluene-ethyl acetate (80:20, v/v)

(E) Dichloromethane-methanol (90:10 v/v)

Vitamin E and Cholesteryl chloroformate were purchased from Fluka. Cholesterol compounds and oligonucleotides were synthesized and characterized in collaboration with Duncan Graham.

DL-α-Tocopheryl-6-O-(2-cyanoethyl-N,N-di-isopropyl) phosphoramidite.[23].

DL-α-tocopherol (1eq; 1g; 2.32mmol) was dried by coevaporation with anhydrous tetrahydrofuran (3 x 10ml) then dissolved in anhydrous tetrahydrofuran (10ml). To this solution was added N,N-diisopropyl-ethylamine (4eq; 9.29mmol; 1.20g; 1.62ml) and 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite (1eq; 2.32mmol; 0.55g; 0.52ml). After 1h the reaction was quenched by the addition of ethanol (1ml) and dichloromethane (100ml) was added. The resulting solution was washed with sat. aqueous $NaHCO_3$ (50ml) and sat. aqueous KCl (3 x 50ml). The organic phase was dried (Na_2SO_4), filtered and solvents were removed by evaporation *in vacuo*. The residue was dissolved in hexane and applied

directly to a wet-flash silica gel chromatography column, eluting with a gradient of 0-10% ethyl acetate in hexane, to give the *title compound* **[23]** as a clear oil (0.8g; 55%). R_f 0.56 (solvent A). δ_P(80MHz; $CDCl_3$) 151.44 (s). f.a.b. ms *m/z* 629.48111 [$(M-H)^+$, $C_{38}H_{66}N_2O_3P$ requires 629.48108].

1-(6-O-DL-α-Tocopheryl)-(8-tetrahydropyranyloxy)-octane. [24]. DL-α-Tocopherol (1eq; 1g; 2.32mmol) was dissolved in dimethyl sulfoxide (25ml) and to this solution was added powdered KOH (4eq; 9.28mmol; 0.52g) and 1-Bromo-8-tetrahydropyranyloxy-octane (1eq; 2.32mmol; 0.68g). After stirring for 2h at 20°C the reaction mixture was poured into water (100ml) and extracted with dichloromethane (3 x 50ml). The resulting solution was washed with water (2 x 25ml) and sat. aqueous KCl (25ml). The organic phase was dried (Na_2SO_4), filtered and solvents removed by evaporation *in vacuo*. The residue was dissolved in hexane containing 0.5% ethyl acetate and applied directly to a wet-flash silica gel chromatography column, eluting with a gradient of 0.5-2% ethyl acetate in hexane, to give the *title compound* **[24]** as a clear oil (1.084g; 73%). R_f 0.39 (solvent B). δ_C(50.320MHz, $CDCl_3$) 11.64 (CH_3); 11.70 (CH_3); 12.58 (CH_3); 19.54 (2xCH_3); 20.52 (CH_2); 20.89 (CH_2); 22.49 (CH_3); 22.57 (CH_3); 23.73 (CH_3); 24.29 (CH_2); 24.67 (CH_2); 25.38 (CH_2); 26.07 (3xCH_2); 27.83 (CH); 29.32 (CH_2); 29.41 (CH_2); 29.60 (CH_2); 30.19 (CH_2); 30.65 (CH_2); 31.16 (CH_2); 32.54 (CH); 32.64 (CH); 37.16 (CH_2); 37.26 (3xCH_2); 39.23 (CH_2); 39.94 (CH_2); 62.16 (CH_2); 67.52 (CH_2); 72.94 (CH_2); 74.56 (C); 98.70 (CH); 117.28 (Ar-C); 122.59 (Ar-C); 125.65 (Ar-C); 127.69 (Ar-C); 147.49 (Ar-C); 148.29 (Ar-C). f.a.b. ms *m/z* 642.55864 [M^+, $C_{42}H_{74}O_4$ requires 642.55868].

1-(6-O-DL-α-Tocopheryl)-octan-8-ol. [25].

To a solution of compound **[24]** (1eq; 1.0g; 1.56mmol) in tetrahydrofuran (20ml) was added conc. aqueous HCl (5ml). After 30h at 20°C the solvents were removed by evaporation *in vacuo* . Traces of water were removed by coevaporation with ethanol, then toluene. The residue was dissolved in dichloromethane and applied directly to a wet-flash silica gel chromatography column, eluting with a gradient of 0-20% ethyl acetate in dichloromethane, to give the *title compound* **[25]** as a clear oil (0.71g; 82%). R_f 0.57 (solvent C). δ_c(50.320MHz, $CDCl_3$) 11.65 (CH_3); 11.73 (CH_3); 12.59 (CH_3); 19.54 (CH_3); 19.62 (CH_3); 20.52 (CH_2); 20.90 (CH_2); 22.51 (CH_3); 22.59 (CH_3); 23.75 (CH_3); 24.31 (CH_2); 24.68 (CH_2); 25.58 (CH_2); 26.04 (CH_2); 27.85 (CH); 29.27 (CH_2); 29.43 (CH_2); 30.17 (CH_2); 31.16 (CH_2); 32.54 (CH); 32.62 (CH_2) 32.67 (CH); 37.15 (CH_2); 37.26 (CH_2); 37.33 (CH_2); 37.44 (CH_2); 39.24 (CH_2); 39.93 (CH_2); 62.87 (CH_2); 72.93 (CH_2); 74.58 (C); 117.30 (Ar-C); 122.60 (Ar-C); 125.65 (Ar-C); 127.68 (Ar-C); 147.49 (Ar-C); 148.21 (Ar-C). f.a.b. ms *m/z* 558.50120 [M^+, $C_{37}H_{66}O_3$ requires 558.50117].

2-cyanoethyl [1-(6-O-DL-α-Tocopheryl)-oct-8-yl] N,N-diisopropylamino phosphoramidite [26].

To a solution of compound **[25]** (1eq; 0.62g; 1.11mmol) in anhydrous tetrahydrofuran (10ml) was added N,N-diisopropylethylamine (4eq; 4.44mmol; 0.57g; 0.77ml) and 2-cyanoethyl-N,N-di-isopropyl-phosphoramidochloridite (1.05eq; 1.17mmol; 0.23g; 0.26ml). After 10min the reaction was quenched by the addition of ethanol (0.5ml) and dichloromethane (100ml) was added. The resulting solution was washed with sat. aqueous $NaHCO_3$ (50ml) and sat. aqueous KCl (3 x 50ml). The organic phase was dried (Na_2SO_4), filtered and solvents were removed by

evaporation *in vacuo*. The residue was dissolved in hexane containing 20% ethyl acetate and applied directly to a wet-flash silica gel chromatography column (which had been pre-equilibrated with hexane containing 20% ethyl acetate and 1% triethylamine), eluting with 20% ethyl acetate in hexane, to give the *title compound* **[26]** as a clear oil (0.781g; 93%) yield. R_f 0.47 (solvent A). δ_P(80MHz; $CDCl_3$) 147.67 (s). f.a.b. ms *m/z* 758.60907 [M^+, $C_{46}H_{83}N_2O_4P$ requires 758.60901].

2,3-Isopropylidenedioxyprop-1-yl *p*-toluenesulphonate [27]

Solketal (1eq; 10g; 76mmol) was dissolved in anhydrous pyridine (100ml), cooled to 0°C and 4-toluene sulphonyl chloride (1eq; 76mmol; 14.43g) was added. The reaction was allowed to warm to 20°C. After 2h the reaction was quenched with methanol (2ml), evaporated to dryness and the residue dissolved in dichloromethane. The resulting solution was washed with sat. aqueous $NaHCO_3$ (50ml), water (2 x 50ml) and sat. aqueous KCl (50ml). The organic phase was dried (Na_2SO_4), filtered and solvents removed by evaporation *in vacuo* to give an oil which solidified on standing. Recrystallized from hexane afforded the *title compound* **[27]** as white waxy crystals (16.052g; 74%). m.p. 41-43°C (*lit.* 41-43°C); R_f 0.49 (solvent D). δ_H(200.130MHz, $CDCl_3$) 1.27 (3H, s, 2-H); 1.30 (3H, s, 2-H); 2.41 (3H, s, Ar-CH_3); 3.68-4.27 (5H, m, 2xCH_2, CH); 7.30 (2H, dd, Ar-3-H, Ar-5-H, J_{ortho}=8.5Hz, J_{meta}=0.7Hz); 7.75 (2H, d, Ar-2H, Ar-6-H, J_{ortho}=8.5Hz). δ_C(50.320MHz, $CDCl_3$) 21.43 (CH_3) ; 24.92 (CH_3); 26.41 (CH_3); 65.89 (CH_2); 69.33 (CH_2); 72.69(CH); 109.81(Ar-C); 127.76 (2xAr-CH); 129.73 (2xAr-CH); 132.36 (C); 144.90 (C). f.a.b. ms *m/z* 287.09529 [$(M+H)^+$, $C_{13}H_{19}O_5S$ requires 287.09531].

1-(6-O-DL-α-Tocopheryl)-propan-2, 3-diol. [28].

DL-α-Tocopherol (1eq; 1.0g; 2.32mmol) was dissolved in dimethyl sulfoxide (25ml). To this solution was added powdered KOH (4eq; 9.28mmol; 0.52g) and compound **[27]** (1.1eq; 2.55mmol; 0.73g). After 24h the reaction mixture was poured into water (100ml) and extracted with dichloromethane (3 x 50ml). The resulting solution was washed with water (50ml) and sat. aqueous KCl (50ml). The organic phase was dried (Na_2SO_4), filtered and solvents removed by evaporation *in vacuo*. The residue was dissolved in hexane containing 0.5% ethyl acetate and applied directly to a wet-flash silica gel chromatography column, eluting with a gradient of 0.5-2% ethyl acetate in hexane. This decolorized the crude product and removed polar impurities. The crude product was evaporated to an oil, then dissolved in tetrahydrofuran (20ml). To this solution was added water (2ml) and conc. aqueous HCl (5ml). After 45min the solvents were removed by evaporation *in vacuo* , and traces of water were removed by coevaporation with ethanol (3 x 10ml) and toluene (10ml). The residue was dissolved in dichloromethane containing 1% methanol and applied directly to a wet-flash silica gel chromatography column, eluting with a gradient of 1-10% methanol in dichloromethane, to give the *title compound* **[28]** as a clear oil (0.63g; 54%). R_f 0.47 (solvent E). δ_c(50.320MHz; $CDCl_3$) 11.66 (2xCH_3); 12.53 (CH_3); 19.55 (CH_3); 19.62 (CH_3); 20.50 (CH_2); 20.89 (CH_2); 22.50 (CH_3); 22.59 (CH_3); 23.70 (CH_3); 24.30 (CH_2); 24.67 (CH_2); 27.85 (CH); 31.08 (CH_2); 32.55 (CH); 32.64 (CH) ; 37.15-37.43 (4xCH_2); 39.23 (CH_2); 39.92 (CH_2); 63.99 (CH_2); 70.89 (CH); 74.03 (CH_2); 74.74 (C); 117.55 (Ar-C); 122.95 (Ar-C); 125.47 (Ar-C); 127.41 (Ar-C); 147.14 (Ar-C); 147.95 (Ar-C). f.a.b. ms *m/z* 504.41782 [M^+, $C_{32}H_{56}O_4$ requires 504.41784].

1-(6-O-DL-α-Tocopheryl)-3-(4,4'-dimethoxytrityloxy)propan-2-ol. [29].

Compound **[28]** (1eq; 0.6g; 1.19mmol) was dried by coevaporation with anhydrous pyridine (3 x 5ml), then dissolved in anhydrous pyridine (7ml) and to this solution was added 4,4'-dimethoxytrityl chloride (1eq; 1.19mmol; 0.40g). After 2h the reaction was quenched with methanol (1ml), and solvents were removed by evaporation *in vacuo*. The residue was dissolved in dichloromethane and the resulting solution was washed with sat. aqueous $NaHCO_3$(30ml) and sat. aqueous KCl (2 x 30ml). The organic phase was dried (Na_2SO_4), filtered and solvents were removed by evaporation *in vacuo*. The residue was dissolved in hexane containing 5% ethyl acetate and applied directly to a wet-flash silica gel chromatography column, eluting with a gradient of 5-10% ethyl acetate in hexane, to give the title compound **[29]** as a pale yellow oil (0.68g; 71%). R_f 0.68 (solvent C). δ_c(50.320MHz, $CDCl_3$) 11.66 (CH_3); 11.76 (CH_3); 12.64 (CH_3); 19.56 (2xCH_3); 20.51 (CH_2); 20.92 (CH_2); 22.52 (CH_3); 22.61 (CH_3); 23.73 (CH_3); 24.32 (CH_2); 24.69 (CH_2); 27.85 (CH); 31.10 (CH_2); 32.57 (CH); 32.65 (CH) ; 37.27 (4xCH_2); 39.24 (CH_2); 39.94 (CH_2); 55.04 (2xDMT-CH_3) 64.27 (CH_2); 70.19 (CH); 73.75 (CH_2); 74.68 (C); 86.01 (DMT-C); 112.98 (4xDMT-CH); 117.44 (Ar-C); 122.79 (Ar-C); 125.63 (Ar-C); 126.66 (DMT-CH); 127.60 (Ar-C); 127.69 (2xDMT-CH); 128.00 (2xDMT-CH); 129.94 (4xDMT-CH); 135.83 (2xDMT-C); 144.69 (DMT-C); 147.41 (Ar-C); 147.79 (Ar-C); 158.35 (2xDMT-C). f.a.b. ms *m/z* 806.54853 [M^+, $C_{53}H_{74}O_6$ requires 806.54851].

1-(6-O-DL-α-Tocopheryl)-3-(4,4'-dimethoxytrityloxy)prop-2-yl hemisuccinate.[30].

To a solution of compound **[29]** (1eq; 0.476g; 0.59mmol) in anhydrous pyridine (10ml) was added succinic anhydride (2.5eq; 1.48mmol; 0.147mg) and 4-dimethylaminopyridine (0.8eq; 0.47mmol; 57mg) in portions over 5 days. The reaction mixture was evaporated to an oil and coevaporated three times with toluene. The residue was taken-up in dichloromethane and washed twice with ice-cold 10% aqueous citric acid. The organic phase was dried (Na_2SO_4), filtered and solvents were removed by evaporation *in vacuo*. The residue was dissolved in dichloromethane containing 50% ethyl acetate and 1% triethylamine and applied directly to a wet-flash silica gel chromatography column, eluting first with dichloromethane:ethyl acetate:NEt_3 49.5:49.5:1 (v/v/v) to elute lipophilic impurities. The the triethylammonium salt of the *title compound* was then eluted from the column with dichloromethane:methanol:NEt_3 89.5:9.5:1 (v/v/v). R_f 0.54 (solvent E).

Procedure for the derivatisation of Long-Chain Alkyl Amino-Controled Pore Glass (CPG) solid support with Compound [8].

Activation of the CPG.

CPG (0.5g) was washed with a 10% solution of N,N-diisopropylethylamine in N,N-dimethylformamide (4x5ml), followed by N,N-dimethylformamide (5x5ml).

Coupling the succinate on to CPG.

To the hemisuccinate **[30]** (0.45 mmol), dissolved in anhydrous dichloromethane (2 ml) was added a solution of 4-nitrophenol (0.45 mmol, 63mg) in anhydrous pyridine (0.1 ml) followed by N,N'-dicyclohexylcarbodiimide (1.125 mmol, 0.23g). After a few minutes

dicyclohexylurea precipitated. After 2.5 h. the dicyclohexylurea was removed by filtration and the filtrate was added to the activated CPG (0.5 g) suspended in N,N-dimethylformamide (2 ml). Triethylamine (0.1 ml) was added and the mixture was agitated with a stream of argon for 48h. The CPG was washed with N,N-dimethylformamide (5x5ml), dichloromethane (5x5ml) and diethyl ether (5x5ml) and dried *in vacuo*.

Capping the CPG.

The functionalised resin was washed with anhydrous tetrahydrofuran (3x5ml) then mixed with anhydrous THF (8 ml), 2M 1-methylimidazole in THF (1.5ml), 1M acetic anhydride in THF (3ml) and agitated for 2.5 h. The CPG was filtered-off, washed successively with THF, dichloromethane and diethyl ether then dried *in vacuo* overnight.

Calculation of the loading of the CPG.

The functionalised CPG (5mg) was detritylated with a solution of trichloroacetic acid in anhydrous dichloromethane in a 10ml volumetric flask. After 5 min. the absorbance of the solution at 504nm was measured to determine the concentration of dimethoxytrityl cations in solution and thus the loading on the CPG.

ε_{504}=76mlcm^{-1}µmol^{-1} for the dimethoxytrityl cation.[64]

The final loading of **[30]** on the CPG was 28.7µmolg^{-1}.

Cholesteryl-3-(carboxyaminohexan-6-ol) [31]

6-Aminohexan-1-ol (1eq; 0.501g; 4.27mmol) was dried by coevaporation with anhydrous pyridine (3 x 5ml) and dissolved in anhydrous pyridine (7ml). To this solution was added a solution of cholesteryl chloroformate (1.906g; 4.24mmol) and anhydrous triethylamine (0.43g; 0.59ml; 4.25mmol) in anhydrous dichloromethane (3ml). The reaction was stirred for 24hrs at 20°C after which dichloromethane (50ml) was added and the

resulting solution washed with sat. aqueous $NaHCO_3$(20ml), water(20ml) and sat. aqueous KCl (2 x 20ml). The organic phase was dried (Na_2SO_4), filtered and solvents were removed by evaporation *in vacuo* . The residue was dissolved in hexane containing 10% ethyl acetate and applied directly to a wet-flash silica gel chromatography column, eluting with a gradient of 0-50% ethyl acetate in hexane, to give the *title compound* **[31]** as a white solid (1.067g; 47%) yield. R_f 0.27 (solvent C). δ_C(50.320MHz, $CDCl_3$) 11.68 (CH_3); 18.54 (CH_3); 19.17 (CH_3); 20.85 (CH_2); 22.40 (CH_3); 22.67 (CH_3); 23.66 (CH_2); 24.11 (CH_2); 25.13 (CH_2); 26.21 (CH_2); 27.84 (CH); 28.05 ($2CH_2$); 29.82 (CH_2); 31.69 (CH, CH_2); 32.38 (CH_2); 35.63 (CH); 35.99 (CH_2); 36.36 (C); 36.80 (CH_2); 38.40 (CH_2); 39.33 (CH_2); 39.54 (CH_2); 40.50 (CH_2); 42.11 (C); 49.79 (CH); 55.92 (CH); 56.48 (CH); 62.46 (CH_2); 74.02 (CH); 122.30 (CH); 139.64 (C); 156.10 (C). f.a.b. ms *m/z* 530.45730 [$(M+H)^+$, $C_{34}H_{60}NO_3$ requires 530.45729].

Cholesteryl-3-carboxyaminohexyl-2-cyanoethyl,N,N-diisopropyl phosphoramidite [32]

Cholesterol-3-(carboxyaminohexan-6-ol) **[31]** (0.499g; 9.44mmol) was dried by coevaporation with anhydrous dichloromethane (3 x 5ml) then dissolved in anhydrous dichloromethane (5ml). Diisopropylammonium tetrazolide (0.081g; 4.72mmol) was added slowly to this solution with stirring until it had all dissolved before adding 2- cyanoethyl N,N,N',N'-tetraisopropyl-phosphorodiamidite (0.31g; 0.33ml; 1.03mmol). After 24h, dichloromethane (50ml) was added and the resulting solution washed with sat. aqueous $NaHCO_3$ (30ml) , sat. aqueous KCl (2 x 30ml) and water (30ml). The organic phase was dried (Na_2SO_4), filtered and the solvent removed *in vacuo* to leave a clear oil. The oil was then dissolved

in hexane containing 10% ethyl acetate and applied directly to a wet-flash silica gel chromatography column (which had been pre-equilibrated with hexane containing 1% triethylamine), eluting with a gradient of 0-25% ethyl acetate in hexane to give the *title compound* **[32]** as a clear oil (0.268g; 38.8%) yield. R_f 0.62 (solvent C). δ_p(80MHz; $CDCl_3$) 147.67 (s). f.a.b. ms *m/z* 729.55730[M^+, $C_{43}H_{76}N_3O_4P$ requires 729.55731].

Cholesteryl-3-(carboxyaminooctan-8-ol) [34]

To a solution of cholesterol chloroformate (0.993g; 2.21mmol) in anhydrous pyridine (5ml) was added 1-amino-8-tetrahydropyranyloxy-octane (synthesis described in Chapter 1 experimental) (0.46g; 2.01mmol). After 15min. at 20°C, dichloromethane (100ml) was added and the resulting solution washed with sat. aqueous $NaHCO_3$ (50ml), water (50ml) and sat. aqueous KCl (2 x 25ml). The organic phase was dried (Na_2SO_4), filtered and solvents were removed by evaporation *in vacuo* . The residue was dissolved in toluene containing 10% ethyl acetate and applied directly to a wet-flash silica gel chromatography column, eluting with a gradient of 0-5% ethyl acetate in toluene, to give a clear oil (1.160g; 90%). A portion of this oil (0.9036g; 1.41mmol) was dissolved in tetrahydrofuran (45ml) and conc. aqueous HCl (0.5ml) and water (4.5ml) were added with stirring. After 24h the reaction was found to be incomplete. The solvent was removed *in vacuo*. Ethanol (2x15ml) was added and removed *in vacuo* before adding THF(45ml), conc. aqueous HCl (0.5ml) and water (4.5ml). This mixture was stirred for a further 30min after which the reaction was found to be complete. The solvent was removed *in vacuo* , ethanol (2x15ml) added and removed *in vacuo* before adding toluene(2x15ml) which was also removed *in vacuo* to remove all traces of water and HCl. The product was then purified by

wet-flash silica gel chromatography eluting with a gradient of 1-50% ethyl acetate in hexane and dried in a vacuum dessiccator to give the title compound **[34]** as a clear glass (0.662g; 83.6%). R_f 0.40 (system C), δ_c(50.320MHz, $CDCl_3$) 11.70 (CH_3); 18.56 (CH_3); 19.18 (CH_3); 20.88 (CH_2); 22.41 (CH_3); 22.67 (CH_3); 23.68 (CH_2); 24.13 (CH_2); 25.50 (CH_2); 26.49 (CH_2); 27.85 (CH); 28.03 (CH_2); 28.07 (CH_2); 29.03 (CH_2); 29.13 (CH_2); 29.56 (CH_2); 29.79 (CH_2); 31.72 (CH, CH_2); 32.49 (CH_2); 35.64 (CH); 36.02 (CH_2); 36.39 (C); 36.83 (CH_2); 38.43 (CH_2); 39.36 (CH_2); 39.57 (CH_2); 40.59 (CH_2); 42.15 (C); 49.83 (CH); 55.97 (CH); 56.52 (CH); 62.60 (CH_2); 74.01 (CH); 122.31 (CH); 139.69 (C); 156.12 (C). f.a.b. ms *m/z* 558.48856 [$(M+H)^+$,$C_{36}H_{63}NO_3$ requires 558.48859].

Cholesteryl-3-carboxyaminooctyl-2-cyanoethyl,N,N-diisopropyl phosphoramidite [35]

Cholesteryl-3-(carboxyaminooctan-8-ol) **[34]** (1eq; 0.574g; 0.995mmol) was dried by coevaporation with anhydrous THF (3 x 15ml) then dissolved in anhydrous THF (15ml). To this solution was added N,N-diisopropylethylamine (4eq; 3.98mmol; 0.513g; 0.71ml) and 2-cyanoethyl-N,N-di-isopropylphosphoramidochloridite (1.1eq; 1.095mmol, 0.252g; 0.237ml). After 15min dichloromethane (50ml) was added to quench the reaction and the resulting solution washed with sat. aqueous $NaHCO_3$(25ml), water (25ml) and sat. aqueous KCl (2 x 25ml). The organic phase was dried (Na_2SO_4), filtered and the solvent removed *in vacuo* to leave a clear oil. The oil was dissolved in hexane containing 10% ethyl acetate and applied directly to a wet-flash silica gel chromatography column (which had been pre-equilibrated with hexane containing 1% triethylamine), eluting with a gradient of 0-10% ethyl

acetate in hexane to give the title compound **[35]** as a clear oil (0.319g; 41%). R_f 0.76 (solvent system C). δ_p(80MHz; $CDCl_3$) 147.95 (s); f.a.b. ms *m/z* 757.58863 [M^+, $C_{45}H_{80}N_3O_4P$ requires 757.58861].

2-cyanoethyl [1-(1-Adamantyl)-eth-2-yl]-N,N-diisopropylamino phosphoramidite [36]

2-(1-Adamantyl)-ethanol (1eq; 0.5g;2.77mmol) was dissolved in anhydrous tetrahydrofuran (10ml) and to this solution was added N,N-diisopropylethylamine (4eq; 0.11mol;1.43g;1.93ml) and 2-cyanoethyl-N,N-di-isopropylphosphoramidochloridite (1.1eq;3.05mmol; 0.72g;0.68ml). The reaction was complete after 2min and was quenched with anhydrous ethanol (1ml), dissolved in dichloromethane and washed with water (40ml) and sat. aqueous KCl (2 x 40ml). The organic phase was dried (Na_2SO_4), filtered and the solvent removed by evaporation *in vacuo*. The residue was purified by wet-flash silica gel chromatography, on a column which had been pre-equilibrated with hexane containing 10% ethyl acetate and 1% triethylamine, eluting with hexane containing 10% ethyl acetate, to give the *title compound* **[36]** as a clear liquid (0.721g; 69%). R_f 0.70 (solvent A). δ_p(145.785MHz; $CDCl_3$) 147.21 (s). f.a.b. ms *m/z* 379.25142 [$(M-H)^+$, $C_{21}H_{36}N_2O_2P$ requires 379.25143].

Synthesis of Lipophilic Oligonucleotides.

Oligonucleotides were prepared on a 1.0 μmole scale on an Applied Biosystems 380B DNA synthesizer using the cyanoethyl phosphoramidite method. Iodine was replaced with tetraethylthiuram disulphide (TETD) for phosphorothioate synthesis. A 15min sulphurization step was used. The phosphoramidite monomers described

above were used at a concentration of 0.1M in anh. dichloromethane. Slightly modified synthesis cycles were used:

(i) A 15 sec delivery of anhydrous dichloromethane to the synthesis column, before and after the coupling step for phosphoramidites **[23]**, **[26]**, **[32]**, **[35]** and **[36]**.

(ii) A 20 sec reverse flush and a 4 sec block flush after each dichloromethane wash.

(iii) The coupling wait time was prolonged to 4 min in order to increase the coupling yield for phosphoramidite **[23]**.

Normal synthesis cycles were used for the synthesis of 3'-Vitamin E oligonucleotides.

Purification of Oligonucleotides.

(i) VitE and Chol-Oligonucleotides.

Buffer A: 0.1M NH_4OAc. *Buffer B:* 0.1M NH_4OAc / 60% MeCN.

Gradient C.

(ii) Adamantyl Oligonucleotides.

Buffer A: 0.1M NH_4OAc. *Buffer B:* 0.1M NH_4OAc / 60% MeCN.

Gradient A.

The final products were purified twice by gel-filtration using Sephadex NAP-10 columns (Pharmacia) and were then lyophilized. Pure water (purified by reverse osmosis) was used for the latter stages of purification.

Ultraviolet Melting Studies

The ultraviolet melting temperatures of lipophilic derivatised S-*rev* hybridised to a normal DNA template with four overhanging bases at both the 3'-, and 5'-end were measured. The ultraviolet melting

temperatures of underivatised S-*rev* hybridised to the same normal DNA template were measured at eight points over a 20-fold concentration range. Each point was measured in triplicate at 260nm on a Perkin-Elmer Lambda 15 ultraviolet spectrometer equipped with a Peltier block and controlled by an IBM PS2 microcomputer. A heating rate of 0.9K per minute was used throughout and the crude data were collected and processed using the PECSS-2 software package. The oligonucleotides were dissolved in a buffer consisting of aqueous sodium chloride (0.1M), sodium dihydrogen orthophosphate (0.01M), sodium cacodylate (0.02M) and EDTA (1mM) which had been adjusted to pH 7.0 by the addition of sodium hydroxide.

Enzyme digests carried out in this UV melting buffer led to incomplete digestion of the oligonucleotide as determined by HPLC. Concentrations of normal oligonucleotide templates were determined by dissolving the sample in a buffer consisting of NaCl (0.1M) and Tris.HCl (10mM) at pH 8.7 and maintaining this at 37°C in the presence of 0.1 mg of phosphodiesterase 1 and 0.1 mg of alkaline phosphatase until complete digestion gave rise to maximum ultraviolet absorbance (< 1 day). Complete digestion was confirmed by HPLC. The published ε_{260} values of the nucleosides were used as standards.[70] As phosphorothioate oligonucleotides are resistant to enzymic digestion, their concentrations were estimated by measuring the U.V. absorbance of the intact oligonucleotide and multiplying this by a factor equivalent to the average proportional increase in U.V. absorbance obtained by digestion of normal oligonucleotides of the same sequence.

D. REFERENCES.

(1) Zamecnik, P.C.; Stephenson, M.L. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 280-284.

(2) Stephenson, M.L.; Zamecnik, P.C. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 285-288.

(3) Agrawal, S. (1992) *Trends in Biotechnology*, **10**, 152-158.

(4) Cohen, J.S. (1992) *Trends in Biotechnology*, **10**, 87-91.

(5) Crooke, S.T. (1992)*Trends in Biotechnology*, **10**, 882-886.

(6) Goodchild, J. (1991) *Antisense Res. Dev.* **1**, 361-364.

(7) Wickstrom, E. (1992) *Trends in Biotechnology*, **10**, 281-287.

(8) Zon, G. (1990) *Annals of the New York Academy of Sciences*, **616**, 161-172.

(9) Uhlmann, E.; Peyman, A. (1990) *Chem. Rev.* **90**, 543-584.

(10) Matteucci, M.D.; Bischofberger, N. (1991) *Ann. Rep. Med. Chem.* **26**, 287-296.

(11) Goodchild, J. (1990) *Bioconjugate Chem.***1**, 165-187.

(12) Quartin, R.S.; Brakel, C.L.; Wetmur, J.G. (1989) *Nucleic Acids Res.* **17**, 7253-7262.

(13) Leonetti, J.P.; Mechti, N.; Degols, G.; Gagnor, C.; Lebleu, B. (1991)*Proc. Natl. Acad. Sci. USA*. **88**, 2702-2706.

(14) Leonetti, J.P.; Mechti, N.; Degols, G.; Lebleu, B. (1991) *Nucleosides and Nucleotides* **10**, 537-539.

(15) Spiller, D.G.; Tidd, D.M. (1992) *Anticancer Drug Design*, **7**, 115-129.

(16) Shoji, Y.; Akhtar, S.; Periasamy, A.; Herman, B.; Juliano, R.L. (1991) *Nucleic Acids Res.* **19**, 5543-5550.

(17) Akhtar, S.; Basu, S.; Wickstrom, E.; Juliano, R.L. (1991) *Nucleic Acids Res.* **19**, 5551-5559.

(18) Verspieren, P.; Cornelissen, A.W.C.A.; Thuong, N.T.; Helene, C.; Toulme, J.J. (1987) *Gene*, **61**, 307-315.

(19) Loke, S.L.; Stein, C.A.; Zhang, X.H.; Mori, K.; Nakanishi, M.; Subasinghe, C.; Cohen, J.S.; Neckers, L.M. (1989)*Proc. Natl. Acad. Sci. USA*. **86**, 3474-3478.

(20) Stein, C.A.; Mori, K.; Loke, S.L.; Subasinghe, C.; Shinozuka, K.; Cohen, J.S.; Neckers, L.M. (1988) *Gene*, **72**, 333-341.

(21) Saison-Behmoaras, T.; Tocque, B.; Rey, I.; Chassignol, M.; Thuong, N.T.; Helene, C. (1991) *The EMBO Journal* , **10**, 111-118.

(22) Shaw, J.P.; Kent, K.; Bird, J.; Fishback, J.; Froehler, B. (1991) *Nucleic Acids Res.* **19**, 747-750.

(23) Bennett, C.F.; Chiang, M.Y.; Chan, H.; Shoemaker, J.E.E.; Mirabelli, C.K. (1992) *Molecular Pharmacology*, **41**, 1023-1033.

(24) Darnell, J.; Lodish, H.; Baltimore, D. (1990) "*Molecular Cell Biology.*", Scientific American Books.

(25) Dautry-Varsat, A.; Lodish, H.F. (1984)*Sci. Am.* **250**, 48-54.

(26) Yakubov, L.A.; Deeva, E.A.; Zarytova, V.F.; Ivanova, E.M.; Ryte, A.S.; Yurchenko, L.V.; Vlassov, V.V. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6454-6458.

(27) Ryte, A.S.; Karamyshev, V.N.; Nechaeva, M.V.; Guskova, Z.V.; Ivanova, E.M.; Zarytova, V.F.; Vlassov, V.V. (1992) *FEBS Lett.* **299**, 124-126.

(28) Boiziau, C.; Toulme, J.J. (1991) *Biochimie*, **73**, 1403-1408.

(29) Goodarzi, G.; Watabe, M.; Watabe, K. (1991) *Biochem. Biophys. Res. Commun.* **181**, 1343-1351.

(30) Miller, P.S.; McParland, K.B.; Jayaraman, K.; Ts'O, P.O.P. (1981) *Biochem.* **20**, 1874.

(31) Kabanov, A.V.; Vinogradov, S.V.; Ovcharenko A.V.; Krivonos, A.V.; Melik-Nubarov, N.S.; Kiselev, V.I.; Severin, E.S. (1990)*Collection of Czech. Chem. Comm.* **55** , 587-589.

(32) Kabanov, A.V.; Vinogradov, S.V.; Ovcharenko A.V.; Krivonos, A.V.; Melik-Nubarov, N.S.; Kiselev, V.I.; Severin, E.S. (1990) *FEBS Lett.* , **259**, 327-330.

(33) Abramova, T.V.; Blinov, V.M.; Vlassov, V.V. (1991) *Nucleosides and Nucleotides*, **10**, 419-422.

(34) Guinosso, C.J.; Hoke, G.D.; Frier, S.; Martin, J.F.; Ecker, D.J.; Mirabelli, C.K.; Crooke, S.T.; Cook, P.D. (1991) *Nucleosides and Nucleotides*, **10**, 259-262.

(35) Brown, M.S.; Goldstein, J.L. (1986) *Science*, **232**, 34-47.

(36) Brown, M.S.; Goldstein, J.L. (1984)*Sci. Am.* **251** (5), 52-60.

(37) Letsinger, R.L.; Zhang, G.; Sun, D.K.; Ikeuchi, T.; Sarin, P.S. (1989)*Proc. Natl. Acad. Sci. USA.* **86**, 6553-6556.

(38) Crews, F.T.; McElhaney, M.R.; Klepner, C.A.; Lippa, A.S. (1988) *Drug Dev. Res.* **14**, 31-44.

(39) Stein, C.A.; Pal, R.; DeVico, A.L.; Hoke, G.; Mumbauer, S.; Kinstler, O.; Sarngadharan, M.G.; Letsinger, R.L. (1991) *Biochemistry,* **30**, 2439-2444.

(40) Farooqui, F.; Sarin, P.; Sun, D.; Letsinger, R.L. (1991) *Bioconjugate Chem.* **2**, 422-426.

(41) Boutorine, A.S.; Boiziau, C.; LeDoan, T.; Toulme, J.J.; Helene, C. (1992) *Biochimie*, **74**, 485-489.

(42) Boutorine, A.S.; LeDoan, T.; Battioni, J.P.; Mansuy, D.; Dupre, D.; Helene, C. (1990) *Bioconjugate Chem.* **1**, 350-356.

(43) Oberhauser, B.; Wagner, E. (1992) *Nucleic Acids Res.* **20**, 533-538.

(44) DeSmidt, P.C.; LeDoan, T.; deFalco, S.; van Berkel, T.J.C. (1991) *Nucleic Acids Res.* **19**, 4695-4700.

(45) Gmeiner, W.H.; Luo, W.; Pon, R.T.; Lown, J.W. (1991) *Bioorganic and Medicinal Chem. Lett.* , **1** , 487-490.

(46) Reed, M.W.; Adams, A.D.; Nelson, J.S.; Meyer, R.B. (1991)*Bioconjugate Chem.* **2** , 217-225.

(47) Manoharan, M.; Guinosso, C.J.; Cook, P.D. (1991) *Tetrahedron Lett.* **32**, 7171-7174.

(48) Shea, R.G.; Marsters, J.C.; Bischofberger, N. (1990) *Nucleic Acids Res.* **18**, 3777-3783.

(49) Schell, P.L. (1974) *Biochim. et Biophys. Acta*, **340**, 323-333.

(50) Lemaitre, M.; Bayard, B.; Lebleu, B. (1987)*Proc. Natl. Acad. Sci. USA.* **84**, 648-652.

(51) Degols, G.; Leonetti, J.P.; Gagnor, C.; Lemaitre, M.; Lebleu, B. (1989) *Nucleic Acids Res.* **17**, 9341-9350.

(52) Stevenson, M.; Iverson, P.L. (1989) *J. General Virology*, **70**, 2673-2682.

(53) Pardridge, W.M.; Boado, R.J. (1991) *FEBS Lett.* **288**, 30-32.

(54) Vestweber, D.; Schatz, G. (1989) *Nature*, **338**, 170-172.

(55) Wagner, E.; Zenke, M.; Cotten, M.; Beng, H.; Birnstiel, M.L. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414.

(56) Behr, J.P.; Demeneix, B.; Loeffler, J.P.; Perez-Mutul, J. (1989) *Proc. Natl. Acad. Sci. USA.*, **86**, 6982-6986.

(57) Felgner, P.L.; Gadek, T.R.; Holm, M., Roman, R.; Chan, H.W.; Wenz, M.; Northrop, J.P.; Ringold, G.M.; Danielson, M. (1987) *Proc. Natl. Acad. Sci. USA.*, **84**, 7413-7417.

(58) Leonetti, J.P.; Machy, P.; Degols, G.; Lebleu, B.; Leserman, L. (1990)*Proc. Natl. Acad. Sci. USA.* **87**, 2448-2451.

(59) Gallo-Torres, H.E. in *Vitamin E . A Comprehensive Treatise .* (1980) Machlin, L.J. (Ed.); Marcel Dekker; New York, 193-267.

(60) Molennaar, I.; Hulstaert, C.E.; Hardonk, M.J. in *Vitamin E . A Comprehensive Treatise.* (1980) Machlin, L.J. (Ed.); Marcel Dekker; New York, 372-389.

(61) Burton, G.W.; Ingold, K.U. (1986) *Acc. Chem. Res.* **19**, 194-201.

(62) Perly, B.; Smith, I.C.P.; Hughes, L.; Burton, G.W.; Ingold, K.U. *Biochim. Biophys. Acta* **1985**, *819* , 131-135.

(63) Benedict, D.R.; Bianchi, T.A.; Cate, L.A. *Synthesis* **1979**, 428-429.

(64) Damha, M.J.; Giannaris, P.A.; Zabarylo, S.V. (1991)*Nucleic Acids Res.* **18**, 3813-3821.

(65) Vu, H.; Hirschbein, B.L. (1991) *Tetrahedron Lett.* **32** , 3005-3008.

(66) Matsukura, M.; Zon, G.; Shinozuka, K.; Robert-Guroff, M.; Shimada, T.; Stein, C.A.; Mitsuya, H.; Wong-Staal, F.; Cohen, J.S.; Broder, S. (1989) *Proc. Natl. Acad. Sci. USA* **86** , 4244-4248.

(67) Wilson, W.D.; Dotrong, M-H.; Zuo, E.T.; Zon, G. (1988) *Nucleic Acids Res.* **16**, 5137-5151.

(68) Aboul-ela, F.; Koh, D.; Tinoco, I.; Martin, F.H. (1985)*Nucleic Acids Res.* **13**, 4811-4824.

(69) Marky, L.A.; Breslauer, K.J. (1987) *Biopolymers,* **26**, 1601-1620.

(70) Sproat, B.S.; Gait, M.J. in "Oligonucleotides-a practical approach" (1985) Gait, M.J. (Ed.), pp109, IRL Press, Oxford.

Appendix 1.

Sequences of oligonucleotides.

DW1 (24mer PCR primer): d(CTC GAG TAT GCC GAG ACC CCT AAT).

A (26mer PCR primer): d(ATG GTT GCA GAA GAT GCC CCT GTT AG).

B (25mer PCR primer): d(CAG TGC AGC ACT CCA CGA GCA GTT C).

D1 (25mer PCR primer): d(TCG TGC CGG CAG TGC CAC TGC ACA C).

D2 (18mer PCR primer): d(CAG ATT GCG ACC CTC CGC).

HC1 (40mer Epstein-Barr virus probe): d(AGC GCG TTT ACG TAA GCC AGA CAG CAG CCA ATT GTC AGT T).

GW1 (24mer PCR primer): d(ATG GTT GCA GAA GAT GCC CCT GTT).

OSWEL1 (23mer PCR primer): d(GTT GAC TCA GAT TGG TTG CAC TT).

OSWEL2 (27mer PCR primer): d(CAT CAG AAT GTC TTG GTC TTC CCC ATT).

S-*rev* (28mer anti-HIV phosphorothioate) S-d(TCG TCG CTG TCT CCG CTT CTT CCT GCC A).

S-*rev* overhanging template (36mer) d(CCT ATG GCA GGA AGA AGC GGA GAC AGC GAC GAA GAC).

Appendix 2.

Relationship between melting temperature and oligonucleotide concentration obtained from thermal denaturation studies on lipophilic phosphorothioate oligonucleotides (S-*rev*).

(a) Unmodified S-*rev*.

Concentration(C) / μM	T_m /K	$1/T_m(x10^3)$ /K^{-1}	ln(C/4)
13.29	341.9	2.925	-12.615
9.48	342.5	2.919	-12.953
5.99	341.9	2.925	-13.412
4.76	341.3	2.929	-13.642
3.58	341.3	2.929	-13.926
2.18	340.3	2.938	-14.422
1.29	340.1	2.940	-14.947
0.604	338.8	2.952	-15.706

(b) Unspaced Vit E-S-*rev.*

Concentration(C) / μM	T_m /K	$1/T_m(x10^3)$ /K^{-1}	ln(C/4)
17.05	344.2	2.906	-12.366
14.02	343.9	2.907	-12.561
11.44	343.9	2.908	-12.765
8.97	343.5	2.911	-13.008
6.14	343.2	2.913	-13.387
3.87	342.7	2.918	-13.849
1.89	342.0	2.924	-14.565
1.27	341.6	2.928	-14.963
0.604	339.4	2.946	-15.706

(c) Octamethylene-spaced Vit E-S-*rev.*

Concentration(C) / μM	T_m /K	$1/T_m(x10^3)$ /K^{-1}	ln(C/4)
19.3	344.5	2.902	-12.242
15.3	343.8	2.909	-12.474
12.5	343.8	2.909	-12.676
9.20	343.7	2.909	-12.983
6.55	343.4	2.912	-13.322
4.44	342.9	2.916	-13.711
2.29	342.1	2.923	-14.373
0.95	341.2	2.930	-15.088

(d) 3'- Vit E-S-*rev.*

Concentration(C) / μM	T_m /K	$1/T_m(x10^3)$ /K^{-1}	ln(C/4)
16.11	343.9	2.908	-12.422
13.93	343.8	2.909	-12.568
11.33	343.6	2.911	-12.774
8.93	343.2	2.913	-13.012
6.80	343.2	2.914	-13.260
4.75	343.2	2.914	-13.644
2.72	342.0	2.924	-14.201
1.31	341.8	2.926	-14.932
0.71	339.7	2.944	-15.541

(e) Hexamethylene-spaced Chol-S-*rev.*

Concentration(C) / μM	T_m /K	$1/T_m(x10^3)$ /K^{-1}	ln(C/4)
15.61	342.8	2.917	-12.454
13.43	342.8	2.917	-12.604
9.93	342.8	2.917	-12.906
7.83	342.4	2.920	-13.144
5.58	342.2	2.922	-13.482
4.16	341.7	2.927	-13.777
1.59	340.6	2.936	-14.734

Appendix 3

Publications.

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The synthesis of oligonucleotides that contain 2,4-dinitrophenyl reporter groups*

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ABSTRACT

The synthesis of non-nucleoside-based phosphoramidites that bear the 2,4-dinitrophenyl group is reported. These labelled phosphoramidites, which have been used in solid-phase oligonucleotide synthesis to attach single and multiple dinitrophenyl groups to the 5′-end of oligonucleotides, are entirely compatible with the normal oligonucleotide synthesis cycle. Multiple labelling can be performed readily in high yield and the resulting oligonucleotides can be purified readily by reversed-phase h.p.l.c. The labelled oligonucleotides have been detected using monoclonal and polyclonal anti-dinitrophenyl antibodies.

INTRODUCTION

Non-radioactive labelling of oligonucleotides has attracted much interest in recent years as the number of biological and biomedical techniques that require labelled oligonucleotides has increased. The major drawback of non-radioactive detection has been its poor sensitivity relative to standard radiolabel-detection techniques. A solution to this problem may lie in the attachment of multiple labelling groups to the oligonucleotide which allow amplification of the detection signal. The attachment of multiple labels must not affect the hybridisation properties and solubility of the oligonucleotide, and it should be controllable in order to allow the optimum number of labels to be attached. The method used for multiple labelling should also be inexpensive and simple.

The most commonly used non-radioactive labelling group is biotin, which has been incorporated into oligonucleotides enzymically[1,2], by reaction with 5′-amino-functionalised oligonucleotides[3–7], and by incorporation of biotinylated phosphoramidites during solid-phase synthesis[8–10]. Only the last technique allows the introduction of multiple labelling groups in a controlled manner. The major disadvantages are that biotin and its analogues are expensive, the synthesis of biotinylated phosphoramidite monomers is made difficult by the poor solubility of biotin, and the presence of high levels of endogenous biotin in certain tissues makes it unsuitable for certain types of hybridisation *in situ*. Enzymic labelling of oligonucleotides with digoxigenin is also possible[11], but this technique is expensive and uncontrolled.

* Dedicated to Professor Grant Buchanan on the occasion of his 65th birthday.
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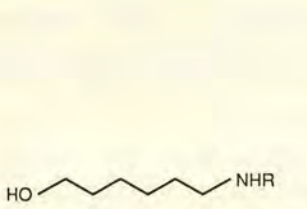
Labelling of oligonucleotides with fluorescent groups has also been achieved[9,12–15]. However, detection of oligonucleotides labelled in this way requires specialised equipment, which is not required in the enzyme-linked immunosorbent assay (ELISA) or chemiluminescent detection of immunogenically labelled oligonucleotides.

An inexpensive labelling group which can be detected immunogenically is the 2,4-dinitrophenyl group (DNP group). The DNP group has been introduced into oligonucleotides *via* the action of deoxynucleotidyl terminal transferase or DNA polymerase on a DNP-aminohexyl derivative of ATP, and by reaction of Sanger's reagent (1-fluoro-2,4-dinitrobenzene) with oligonucleotides that contain an aminohexyl derivative of adenosine[16]. DNP groups have been introduced photochemically[17] and by reaction with brominated bases[18]. Multiple DNP groups have been incorporated into oligonucleotides in a more controlled manner using a DNP nucleoside phosphoramidite during solid-phase synthesis[9]. The DNP group is unstable under the normal conditions for base-catalysed deprotection, but this problem can be overcome by using labile base-protected monomers for DNA synthesis.

The aim of the present work is to develop simple DNP phosphoramidites for the mono- or poly-labelling of oligonucleotides during solid-phase synthesis. The strategy used involves inexpensive, readily available, non-nucleoside starting materials that can be functionalised in the minimum number of steps to give the desired DNP phosphoramidite, which can then be used in standard procedures to give the desired mono- or poly-labelled oligonucleotides.

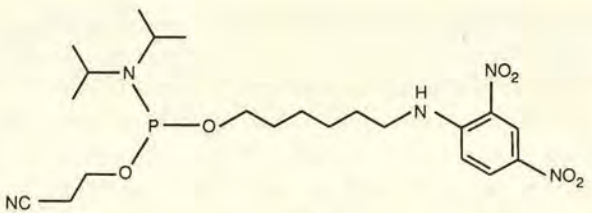
RESULTS AND DISCUSSION

Synthesis of a mono-labelling DNP phosphoramidite. — The DNP phosphoramidite **3**, which allows the attachment of a single DNP group to the 5′-end of oligonucleotides during solid-phase synthesis, was obtained as follows. 6-Aminohexan-1-ol (**1**) was treated with Sanger's reagent in methanol to give the mono-DNP derivative **2**. The reaction of **2** with 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite then gave **3** (80%).



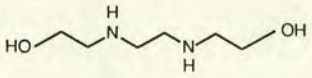
1 R = H

2 R = DNP



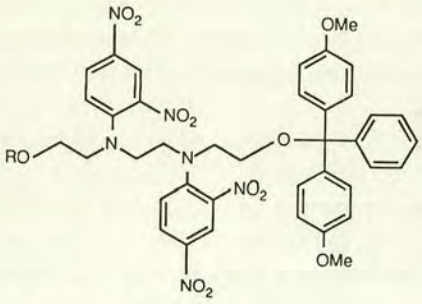
3

Synthesis of a poly-labelling DNP phosphoramidite. — The DNP phosphoramidite **7**, which allows the attachment of multiple DNP groups to oligonucleotides during solid-phase synthesis, was obtained as follows. 3,6-Diazaoctane-1,8-diol (**4**) was treated with Sanger's reagent in methanol and the product (**5**, 81%) yielded crystals that were suitable for X-ray diffraction analysis. The X-ray crystal structure of **5** showed that the DNP groups were arranged "*anti*" with respect to each other[19]. If the same conformation is retained in solution, this may be advantageous, allowing the binding of an antibody to each of the two DNP groups, thus enhancing the detection sensitivity. The reaction of **5** with 4,4′-dimethoxytrityl chloride in pyridine gave **6a** (31%). The poor yield was due to the equivalence of the two primary hydroxyl functions present. This reaction was not optimised, but **5** could be recovered by treatment of the bis(4,4′-dimethoxytrityl) side-product **6b** with acetic acid. Reaction of **6a** with 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite gave **7** (84%). One advantage of **7** is that it allows the attachment of two DNP groups to the oligonucleotide per synthesis cycle.



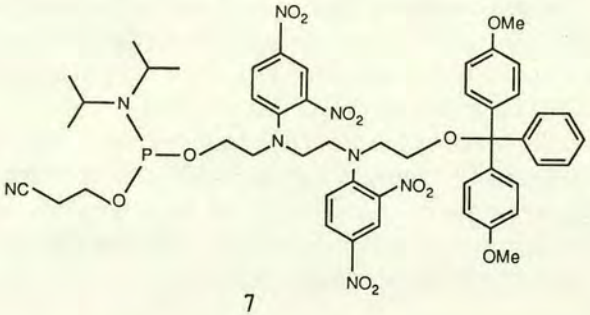
4 R = H

5 R = DNP



6a R = H

6b R = 4,4′-dimethoxytrityl



7

Synthesis of DNP-labelled oligonucleotides. — The DNP phosphoramidites **3** and **7** were used as 0.15M solutions in anhydrous acetonitrile and anhydrous dichloromethane, respectively, for the solid-phase synthesis of labelled oligonucleotides. A standard 0.2-μmol-scale synthesis cycle was used. The coupling efficiency of **7** was >97% in all syntheses. The coupling efficiency of **3** could not be measured directly, but was estimated by h.p.l.c. analysis to be >>90%. Oligonucleotides that had up to ten DNP groups (*i.e.*, five couplings of **7**) were synthesised, although, theoretically, any even number of DNP groups could be attached in this way.

Stability of the labelling to basic deprotection conditions. — The single DNP label introduced by the coupling of **3** to the 5'-end of the oligonucleotide was found by h.p.l.c. to be stable to normal base-catalysed deprotection (5 h, 55°, conc. NH_4OH). However, the multiple labels introduced by the coupling of **7** to the 5'-end of oligonucleotides were unstable under these conditions. H.p.l.c. showed that there was only one degradation product formed, which was neither 2,4-dinitroaniline nor 2,4-dinitrophenol. This instability problem was overcome by the use of commercially available phosphoramidites for DNA synthesis (Pharmacia "PAC"-amidites or ABI "FOD"-amidites), which bear base protecting groups that are removed under milder conditions (5 h in conc. NH_4OH at 20°). Under such conditions, the DNP label was degraded only to a very small extent, and no problems arose in the purification and isolation of the labelled oligonucleotide in high yield.

Purification of DNP-labelled oligonucleotides. — The highly lipophilic nature of the DNP group results in DNP-labelled oligonucleotides being eluted later than unlabelled failure sequences on reversed-phase h.p.l.c. This difference makes the purification of DNP-labelled oligonucleotides extremely easy, as the labelled oligonucleotide behaves similarly to a conventional "trityl-on" sequence. The effect of increasing the number of DNP labels on the time of elution is shown in Fig. 1. The labelled oligonucleotides are bright yellow in colour, which allows them to be distinguished readily from unlabelled sequences. Traces of the low-molecular-weight degradation product were removed when the sample was passed through a column of Sephadex G25 during the normal purification procedure.

Detection of DNP-labelled oligonucleotides. — Preliminary experiments confirmed that anti-DNP antibodies bind to DNP-labelled oligonucleotides, and that the antibodies can be detected both on the basis of radioactivity and by an ELISA method. Oligonucleotides labelled with one, two, six, and ten DNP groups were fixed on nylon filters in duplicate by u.v. irradiation. The filters were blocked using bovine serum albumin with EDTA to inhibit any DNAase activity, washed, and incubated with a 1/200 dilution of K3 monoclonal anti-DNP antibody for 1 h. After further washing, one filter was treated with ^{125}I-Protein A and then washed, and the signal was detected using X-ray film. The other filter was incubated with an anti-mouse-IgG-peroxidase conjugate for 1 h, washed, and detected by 4-chloro-1-naphthol and H_2O_2, which gave a blue precipitate. Further experiments are in progress.

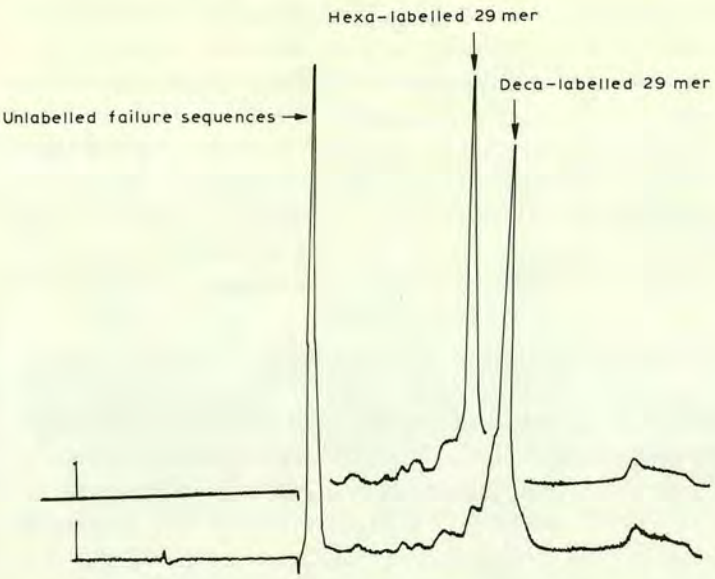


Fig. 1. Superimposed traces from h.p.l.c. of identical 29mers labelled with six (upper trace) and ten (lower trace) DNP groups, showing the effect of the lipophilicity of the DNP groups on elution time. Buffer *A*, 0.1M NH_4OAc; buffer *B*, 0.1M NH_4OAc/60% of acetonitrile.

EXPERIMENTAL

All solvents were of analytical grade. Alcohol-free anhydrous dichloromethane was prepared by distillation from CaH_2, and anhydrous tetrahydrofuran by distillation from sodium–benzophenone. Hexane was dried over sodium wire. Anhydrous acetonitrile was supplied by Applied Biosystems Inc. ^{1}H-N.m.r. spectra were recorded with Bruker WP-80 (80 MHz) and WP-200 (200 MHz) spectrometers. ^{31}P-N.m.r. spectra (81 MHz) were recorded with the latter spectrometer. F.a.b. mass spectra (positive ion, thioglycerol matrix) were recorded on a Kratos MS50 TC spectrometer. Oligonucleotide synthesis was performed with an Applied Biosystems 381A DNA synthesiser, and ending procedures with an ABI 380B DNA synthesiser. 2-Cyanoethyl phosphoramidite monomers for DNA synthesis were supplied by Applied Biosystems and Pharmacia. Sephadex G-25 N.A.P. (nucleic acid purification) columns were supplied by Pharmacia. Flash-column chromatography was carried out on Silica Gel 60 (Fluka), and t.l.c. on Silica Gel 60 F_{254} (Merck), using *A*, toluene–ethyl acetate (1:1), 1% of triethylamine; *B*, toluene–ethyl acetate (4:1), 1% of triethylamine; *C*, dichloromethane–methanol (9:1), 1% of triethylamine; *D*, dichloromethane–methanol (99:1), 1% of triethylamine; *E*, dichloromethane–2-propanol (95:5), 1% of triethylamine; *F*, dichloromethane–ethyl acetate (1:1), 1% of triethylamine.

6-(2,4-Dinitrophenylamino)hexan-1-ol (**2**). — To a solution of 6-aminohexan-1-ol (0.8 g, 6.8 mmol) in methanol (20 mL) was added 1-fluoro-2,4-dinitrobenzene (1.3 g, 1 equiv.). After 12 h, the solvent was evaporated *in vacuo* and the resulting oil was washed with ether to leave crude **2** (1.3 g, 70%). Recrystallisation from ether–methanol gave **2** (1.04 g, 54%), m.p. 75°, R_F 0.52 (solvent *C*). F.a.b. mass spectrum: *m/z* 284.12462 [calc. for $C_{12}H_{18}N_3O_5$: $(M^+ + H)$ *m/z* 284.12463]. ^{1}H-N.m.r. data ($CDCl_3$): δ 1.1–2.0 (m, 9 H, 4 CH_2 and OH), 3.25–3.5 (m, 2 H, H-1,1), 3.64 (t, 2 H, *J* 6.2 Hz, H-6,6), 6.9 (d, 1 H, Ar H-6), 8.25 (dd, 1 H, Ar H-5), 8.5 (bs, 1 H, NH), 9.1 (d, 1 H, Ar H-3).

Anal. Calc. for $C_{12}H_{17}N_3O_5$: C, 50.88; H, 6.01; N, 14.84. Found: C, 50.40; H, 5.78; N, 14.70.

2-Cyanoethyl 6-(2,4-dinitrophenylamino)hexyl N,N-*di-isopropylphosphoramidite* (**3**). — To a solution of **2** (140 mg, 0.495 mmol) in anhydrous tetrahydrofuran (5 mL) was added anhydrous *N,N*-di-isopropylethylamine (0.1 mL, 1 equiv.) and 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite (0.132 mL, 1.2 equiv.). After 1 h, the reaction was quenched by the addition of ethyl acetate (20 mL), and the solution was washed with aqueous $NaHCO_3$ (2 × 5 mL) and brine (2 × 5 mL), dried (Na_2SO_4), filtered, and evaporated *in vacuo*. Flash-column chromatography (dichloromethane–ethyl acetate, 1:1) of the residue gave an oil which was dissolved in dichloromethane and precipitated with anhydrous hexane to give **3** (100 mg, 80%) as an oil, R_F 0.8 (solvent *F*). ^{31}P-N.m.r. data: δ 147.54 (s).

1-(4,4'-Dimethoxytrityloxy)-6-(2,4-dinitrophenylamino)hexane. — To a solution of **2** (1 g, 3.53 mmol) in anhydrous pyridine (15 mL) was added 4,4'-dimethoxytrityl chloride (1 equiv., 1.2 g). The mixture was protected from moisture and the reaction was complete after 1 h at 20°. Water (150 mL) was added and the product was extracted into chloroform (3 × 30 mL). The combined extracts were dried (Na_2SO_4) and filtered, and the solvent was evaporated *in vacuo*. The product was purified by flash-column chromatography (dichloromethane + 2% of triethylamine) to give the title compound isolated as an oil (1.24 g, 60%), R_F 0.78 (solvent *C*); F.a.b. mass spectrum: *m/z* 585.24744 (calc. for $C_{33}H_{35}N_3O_7$: *m/z* 585.24748). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.0–3.5 (m, 12 H, 6 CH_2), 3.75 (s, 6 H, 2 OMe), 6.8 (m, 4 H, Ar), 7.2–7.5 (m, 9 H, Ar and DNP H-6), 8.25 (d, 1 H, DNP H-5), 8.5 (bs, 1 H, NH), 9.1 (d, 1 H, DNP H-3).

3,6-Bis(2,4-dinitrophenyl)-3,6-diazaoctane-1,8-diol (**5**). — To a solution of 3,6-diazaoctane-1,8-diol (**4**; 1.5 g, 10 mmol) in methanol (30 mL) was added triethylamine (2.5 equiv., 2.53 g, 3.48 mL, 25 mmol) and 1-fluoro-2,4-dinitrobenzene (2 equiv., 3.72 g, 20 mmol). After 3.5 h, the mixture was cooled to 0°, and the orange precipitate was collected, washed with methanol (30 mL) and ether (3 × 30 mL), and dried *in vacuo* to give **5** (3.89 g, 81%). The product was recrystallised three times from methanol to give **5** with m.p. 149–150°, R_F 0.38 (solvent *C*). F.a.b. mass spectrum: *m/z* 481. ^{1}H-Nm.r. data ($CDCl_3$): δ 3.0–4.0 (m, 12 H, 6 CH_2), 4.7 (t, 2 H, 2 OH), 7.33 (d, 2 H, $J_{5,6}$ 9.5 Hz, DNP H-6), 8.13 (dd, 2 H, $J_{3,5}$ 2.8 Hz, DNP H-5), 8.47 (d, 2 H, DNP H-3).

Anal. Calc. for $C_{18}H_{20}N_6O_{10}$: C, 45.0; H, 4.16; N, 17.5. Found: C, 44.8; H, 4.18; N, 17.4.

8-(4,4'-Dimethoxytrityloxy)-3,6-bis(2,4-dinitrophenyl)-3,6-diazaoctan-1-ol (**6a**). — Anhydrous pyridine (3 × 30 mL) was evaporated from **5** (3.30 g, 6.88 mmol), which was then dissolved in anhydrous pyridine (60 mL). To this solution was added dropwise, during 30 min, a solution of 4,4'-dimethoxytrityl chloride (1.3 equiv., 3.03 g, 8.94 mmol) in anhydrous pyridine (100 mL). After 2 h, more 4,4'-dimethoxytrityl chloride (0.2 equiv., 0.47 g, 1.38 mmol) in anhydrous pyridine (20 mL) was added dropwise during 20 min and, after a further 30 min, the reaction was quenched with methanol (20 mL) and the solvent was evaporated *in vacuo*. A solution of the residue in dichloromethane (250 mL) was washed with saturated aqueous $NaHCO_3$ (50 mL) and water (3 × 50 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Column chromatography (solvent *A*) of the residue gave amorphous **6a** (1.68 g, 31%), R_F 0.29 (solvent *D*), 0.58 (solvent *E*), and 0.35 (solvent *A*). F.a.b. mass spectrum: *m/z* 783.26259 [calc. for $C_{39}H_{39}N_6O_{12}$: $(M^+ + H)$ *m/z* 783.26257]. ^{1}H-N.m.r. data ($CDCl_3$): δ 2.0–2.25 (bs, 1 H, OH), 3.25–3.72 (m, 12 H, 6 CH_2), 3.73 (s, 6 H, 2 OCH_3), 6.65–7.23 (m, 15 H, Ar and DNP H-6), 8.06 (dd, 1 H, $J_{5,6}$ 9.3, $J_{3,5}$ 2.7 Hz, DNP H-5), 8.13 (dd, 1 H, DNP H-5), 8.52 (d, 1 H, DNP H-3), 8.55 (d, 1 H, DNP H-3).

2-Cyanoethyl [8-(4,4'-dimethoxytrityloxy)-3,6-bis(2,4-dinitrophenyl)-3,6-diazaoctyl] N,N-*di-isopropylphosphoramidite* (**7**). — Anhydrous tetrahydrofuran (3 × 20 mL) was evaporated from **6a** (0.643 g, 0.822 mmol) which was then dissolved in anhydrous tetrahydrofuran (30 mL). To this solution was added *N,N*-di-isopropylethylamine (4 equiv., 3.29 mmol, 0.425 g, 0.573 mL) and 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite (1.5 equiv., 1.23 mmol, 0.291g, 0.276 mL). The solution was stirred at 20° for 15 min, quenched with ethyl acetate (30 mL), washed with brine (4 × 100 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Column chromatography (solvent *B*) of the residue gave **7** as an oil, a solution of which, in the minimum volume of anhydrous dichloromethane, was added dropwise to anhydrous hexane (750 mL) at −78°. The orange precipitate which formed was collected and washed with anhydrous hexane to give **7** (0.678 g, 84%), R_F 0.50 (solvent *B*). F.a.b. mass spectrum: *m/z* 983.37044 [calc. for $C_{48}H_{56}N_8O_{13}P$: $(M^+ + H)$ *m/z* 983.37042]. ^{31}P-N.m.r. data ($CDCl_3$): δ 149.12 (s).

Synthesis of DNP-labelled oligonucleotides. — Oligonucleotide synthesis was performed with an Applied Biosystems 381A DNA synthesiser, using the standard 0.2-μmol-scale synthesis cycle. For the additions to the 5'-end of oligonucleotides, a 0.15M solution of **3** in anhydrous acetonitrile and a 0.15M solution of **7** in anhydrous dichloromethane were used. Ending procedures were carried out on an Applied Biosystems 380B DNA synthesiser.

Degradation of 7-CTC GAG TAT GCC GAG ACC CCT AAT with ammonia. — A solution of 6 o.d. units (A_{264}) of the title compound in distilled water (200 μL) was added to conc. ammonia (1.8 mL) in a sealed vial. Every hour, an aliquot (200 μL) was removed, diluted with 0.1M NH_4OAc (700 μL), and analysed by h.p.l.c.

Measurement of monomer coupling efficiencies. — Coupling efficiencies were measured by comparison of the absorbance at 498 nm of the 4,4'-dimethoxytrityl cations produced in the deprotection steps of successive synthesis cycles. Each fraction was diluted to 25 mL with 0.1M toluene-4-sulphonic acid in acetonitrile.

H.p.l.c. — A Gilson 303 and a Perkin–Elmer 410 system were used with ABI aquapore octyl reversed-phase columns. The buffer systems were varied according to the number of DNP labels attached to the oligonucleotide. The general buffer systems were *A*, 0.1M NH_4OAc; and *B*, 0.1M NH_4OAc/X% of acetonitrile where $30\% < X < 70\%$. The greater the number of DNP groups present, the higher the percentage of acetonitrile used in buffer *B*. These buffers were used (flow rate, 3 mL/min) in combination with the following gradient to achieve the optimum separation for the sequence being examined:

Time (min)	0	3	4	26	28	29	32
Buffer *B* (%)	0	0	15	100	100	0	0

ACKNOWLEDGMENTS

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REFERENCES

1 J. J. Leary, D. J. Brigati, and D. C. Ward, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 4045–4049.
2 P. R. Langer, A. A. Waldrop, and D. C. Ward, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 6633–6637.
3 A. F. Cook, E. Vuocolo, and C. L. Brakel, *Nucleic Acids Res.*, 16 (1988) 4077–4095.
4 R. P. Viscidi, C. J. Connelly, and R. H. Yolken, *J. Clin. Microbiol.*, 23 (1986) 311–317.
5 Applied Biosystems User Bulletin No. 49 (1988).
6 L. Wachter, J. Jablonski, and K. L. Ramachandran, *Nucleic Acids Res.*, 14 (1986) 7985–7994.
7 S. Agrawal, C. Christodoulou, and M. J. Gait, *Nucleic Acids Res.*, 14 (1986) 6227–6245.
8 A. M. Alves, D. Holland, and M. D. Edge, *Tetrahedron Lett.*, 30 (1989) 3089–3092.
9 A. Roget, H. Bazin, and R. Teoule, *Nucleic Acids Res.*, 17 (1989) 7643–7651.
10 K. Misiura, I. Durrant, M. R. Evans, and M. J. Gait, *Nucleic Acids Res.*, 18 (1990) 4345–4354.
11 K. J. Hillan, M. Farquharson, R. Harvie, T. A. McKee, R. N. M. MacSween, and A. M. McNicol, *J. Histochem. Cytochem.*, (in press).
12 F. Schubert, K. Ahlert, D. Cech, and A. Rosenthal, *Nucleic Acids Res.*, 18 (1990) 3427.
13 M. S. Urdea, B. D. Warner, J. A. Running, M. Stempien, J. Clyne, and T. Horn, *Nucleic Acids Res.*, 16 (1988) 4937–4956.
14 L. M. Smith, J. Z. Sanders, R. J. Kaiser, P. Hughes, C. Dod, C. R. Connell, C. Heiner, S. B. H. Kent, and L. E. Hood, *Nature (London)*, 321 (1986) 674–678.
15 S. Agrawal and P. C. Zamecnik, *Nucleic Acids Res.*, 18 (1990) 5419–5423.
16 C. Vincent, P. Tchen, M. Cohen-Solal, and P. Kourilsky, *Nucleic Acids Res.*, 10 (1982) 6787–6796.
17 G. H. Keller, D. Huang, and M. M. Manak, *Anal. Biochem.*, 177 (1989) 392–395.
18 G. H. Keller, D. Huang, M. M. Manak, and R. Ting, *Anal. Biochem.*, 170 (1988) 441–450.
19 G. A. Leonard, personal communication.

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Attachment of Vitamin E Derivatives to Oligonucleotides during Solid-Phase Synthesis.

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Keywords: Antisense; phosphorothioate; oligonucleotide; Vitamin E; tocopherol.

Abstract: Compounds have been synthesized which allow the attachment of Vitamin E to the 5'-, and 3'-ends of oligonucleotides, and the attachment of a Vitamin E moiety on an octamethylene spacer to the 5'-end, all during solid-phase synthesis. The effects on cellular uptake, distribution, and antiviral activity of the attachment of the extremely lipophilic Vitamin E moiety to phosphorothioate 'antisense' oligonucleotides are now being investigated.

The use of antisense oligonucleotides as drugs to inhibit viral replication in cells has attracted great interest in recent years as the potential of this strategy has become apparent[1]. Some of the problems inherent in such a strategy have been solved but many difficulties remain. One of the major problems encountered is the low permeability of cell membranes to large, polyionic oligonucleotides. Attempts to increase the cellular uptake of antisense oligonucleotides by the conjugation of lipophilic molecules are well known. Such lipophilic molecules fall into two main groups:

(i) Those known to interact specifically with cell membranes. *eg.* cholesterol[2-5] and certain lipids[6].

(ii) Those which impart general lipophilicity to the oligonucleotide. *eg.* alkyl chains[7,8].

Vitamin E (α-tocopherol) is an attractive candidate as a lipophilic carrier for the following reasons:

(1) It is inexpensive and essentially non-toxic.

(2) It has only one functional group for derivatization, the rest of the molecule is chemically inert.

(3) It is found mainly in association with membranes of subcellular organelles[9], such as the endoplasmic reticulum and mitochondria, rather than in the plasma membrane. This maximizes the possibility of intracellular transport.

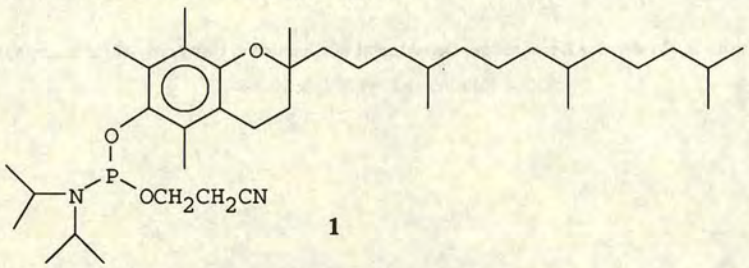
(4) In membranes, the phytyl chain of the Vitamin E is embedded in the membrane, with the phenolic hydroxyl group towards the surface[10,11]. Thus attachment of the oligonucleotide to the phenolic hydroxyl group should have a minimal effect on the interactions of the Vitamin E with membranes.

Here we describe the synthesis of molecules which allow the attachment of Vitamin E to the 5'-, and 3'-ends of oligonucleotides, and the attachment of a Vitamin E moiety *via.* an octamethylene spacer to the 5'-end, during solid-phase synthesis. In all experiments DL-α-tocopherol was used.

(1) Synthesis of Vitamin E derivatives.

(a) Unspaced Vitamin E Phosphoramidite **1**.(Scheme 1)

This was prepared by phosphitylation of Vitamin E with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite and diisopropylethylamine in THF.



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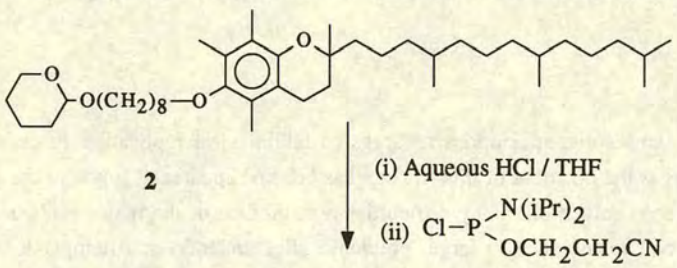
Scheme 1

(b) Octamethylene-spaced Vitamin E phosphoramidite **3** (Scheme 2).

Vitamin E was reacted with commercially available 1-Bromo-8-tetrahydropyranyloxyoctane (Lancaster) in DMSO in the presence of powdered KOH [12] to form the asymmetric ether **2**. The THP protecting group was removed by treatment with aqueous HCl in THF, and the resulting alcohol was phosphitylated.



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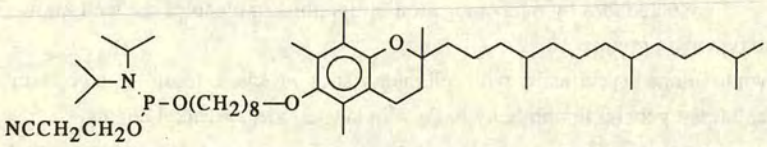
(i) Aqueous HCl / THF

(ii) Cl—P(N(iPr)2)(OCH2CH2CN)



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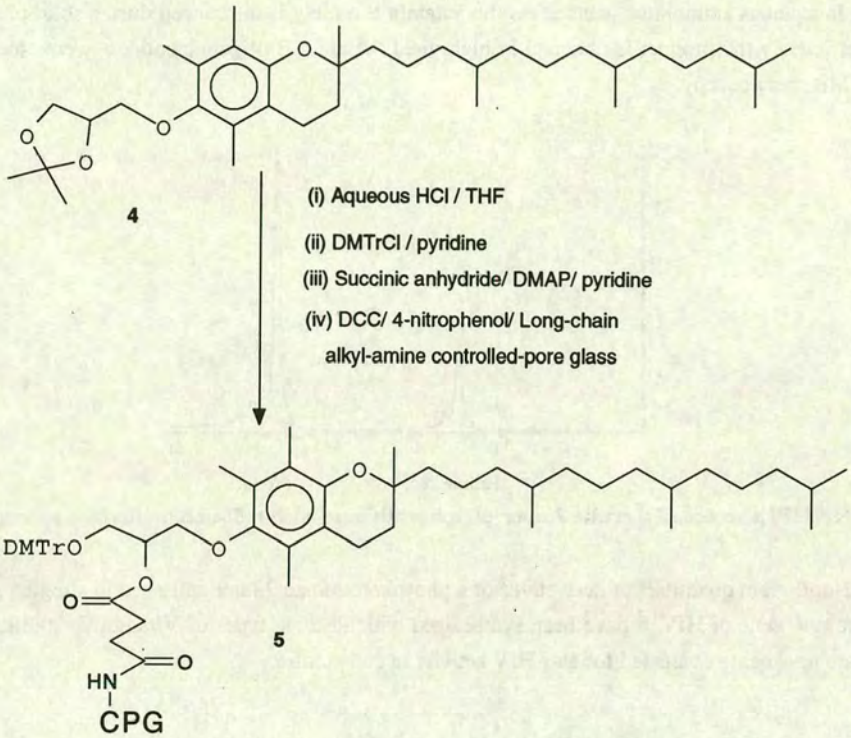
Scheme 2

(c) Vitamin E-derivatized Controlled PoreGlass (CPG), **5** (Scheme 3)

Solketal was reacted with 4-toluene sulphonyl chloride in pyridine. The resulting tosyl solketal was reacted with Vitamin E in DMSO in the presence of powdered KOH [12] to form the asymmetric ether **4**. The

acetonide protecting group was removed by treatment with aqueous HCl in THF, and the resulting alcohol was reacted with dimethoxytrityl chloride in pyridine. The remaining secondary alcohol was then reacted with succinic anhydride/ DMAP in pyridine. The resulting tritylated-succinylated Vitamin E solketal derivative was coupled to LCAA-CPG to give **5** with a loading of 24 $\mu mol.g^{-1}$.



Scheme 3

(2) Oligo ucleotide Synthesis.

Normal and phosphorothioate[13] Vitamin E derivatised oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer.

For 3'-Vitamin E oligonucleotides:

- standard DNA and phosphorothioate synthesis cycles were used.

For 5'-Vitamin E oligonucleotides:

-Phosphoramidites **1** and **3** were insoluble in acetonitrile and were used as 0.1*M* solutions in anhydrous dichloromethane,

-Minor alterations to the synthesis cycles were required (the synthesis columns were washed with anhydrous dichloromethane before and after the coupling reaction to prevent precipitation).

-Coupling efficiencies were >95% estimated by HPLC.

Vitamin E oligonucleotides were stable in conc. aqueous ammonia at 55°C for >24h (HPLC analysis).

(3) Oligonucleotide Purification.

The extreme lipophilicity of Vitamin E oligonucleotides greatly facilitates reversed-phase HPLC (RP-HPLC) purification, the products eluting approx. 15min later than all failure sequences in a gradient of acetonitrile in aqueous ammonium acetate. As the Vitamin E moiety is introduced during solid-phase synthesis with high efficiency the product is obtained in high yield. Vitamin E oligonucleotides were characterized by 600MHz NMR spectroscopy.

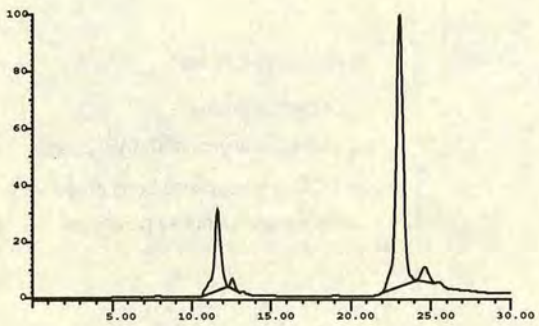


Figure 1: **RP-HPLC trace of a crude 28mer phophorothioate with a 5'-octamethylene spaced Vitamin E attached.**

Multi-milligram quantities of derivatives of a phosphorothioate 28mer antisense to a region near the start codon of the *rev* gene of HIV[14] have been synthesized with all three types of Vitamin E modification. These sequences are now being evaluated for anti-HIV activity in cell culture.

References.

1. Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543-584.
2. Gmeiner, W.H.; Luo, W.; Pon, R.T.; Lown, J.W. *Bioorganic and Medicinal Chem. Lett.* **1991**, *1*, 487-490.
3. Reed, M.W.; Adams, A.D.; Nelson, J.S.; Meyer, R.B. *Bioconjugate Chemistry* **1991**, *2*, 217-225.
4. Letsinger, R.L.; Zhang, G.; Sun, D.K.; Ikeuchi, T.; Sarin, P.S. *Proc. Natl. Acad. Sci. USA.* **1989**, *86*, 6553-6556.
5. Boutorin, A.S.; Gus'kova, L.V.; Ivanova, E.M.; Kobetz, N.D.; Zarytova, V.F.; Ryte, A.S.; Yurchenko, L.V.; Vlassov, V.V. *FEBS Lett.* **1989**, *254*, 129-132.
6. Shea, R.G.; Marsters, J.C.; Bischofberger, N. *Nucleic Acids Res.* **1990**, *18*, 3777-3783.
7. Saison-Behmoaras, T.; Tocque, B.; Rey, I.; Chassignol, M.; Thuong, N.T.; Helene, C. *The EMBO Journal* **1991**, *10*, 111-118.
8. Kabanov, A.V.; Vinogradov, S.V.; Ovcharenko A.V.; Krivonos, A.V.; Melik-Nubarov, N.S.; Kiselev, V.I.; Severin, E.S. *FEBS Lett*. **1990**, *259*, 327-330.
9. Molennaar, I.; Hulstaert, C.E.; Hardonk, M.J. in *Vitamin E. A Comprehensive Treatise*. Machlin, L.J. (Ed.); Marcel Dekker; New York, **1980**, 372-389.
10. Burton, G.W.; Ingold, K.U. *Acc. Chem. Res.* **1986**, *19*, 194-201.
11. Perly, B.; Smith, I.C.P.; Hughes, L.; Burton, G.W.; Ingold, K.U. *Biochim. Biophys. Acta* **1985**, *819*, 131-135.
12. Benedict, D.R.; Bianchi, T.A.; Cate, L.A. *Synthesis* **1979**, 428-429.
13. Vu, H.; Hirschbein, B.L. *Tetrahedron Lett.* **1991**, *32*, 3005-3008.
14. Matsukura, M.; Zon, G.; Shinozuka, K.; Robert-Guroff, M.; Shimada, T.; Stein, C.A.; Mitsuya, H.; Wong-Staal, F.; Cohen, J.S.; Broder, S. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 4244-4248.

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Synthesis and physical properties of *anti*-HIV antisense oligonucleotides bearing terminal lipophilic groups

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ABSTRACT

A number of phosphoramidite monomers have been prepared and used in the synthesis of antisense phosphorothioate oligonucleotides bearing 5′-polyalkyl and cholesterol moieties. Similar groups have also been attached to the 3′-end of oligonucleotides by means of functionalised CPG. Melting temperatures of duplexes formed between phosphorothioate oligonucleotides with lipophilic end-groups and complementary DNA strands were found to be identical to those formed by the equivalent unmodified phosphorothioates.

INTRODUCTION

The use of antisense oligonucleotides and their analogues as highly specific inhibitors of gene expression and viral replication has attracted great interest in recent years (1, 2, 3). It has been possible to demonstrate reasonable inhibition in some cases, but the very high activity necessary for potential drug candidates has proved elusive. One of the major limitations is the low permeability of cell membranes to large polyanionic oligonucleotides. Cellular uptake of oligonucleotides has been shown to be poor (4) and this has been improved by the attachment of lipophilic groups that interact specifically with cell membranes, *ie:* cholesterol (5–9), vitamin E (10) and the 1,2-di-O-hexadecyl-3-glyceryl group (11); or those which impart general lipophilicity to the oligonucleotide, *eg.* hydrocarbon chains (12,13). In addition to the antisense effect (7,11,12,13), oligonucleotides (18), particularly those bearing lipophilic groups, have been shown to be active as antiviral agents in a non-sequence-dependent manner (7,8,11). The origin of this interesting effect is not well understood, but may arise by interactions of oligonucleotides with polymerases such as viral reverse transcriptases (7,8). An alternative explanation is that oligonucleotides interact with the surfaces of cells or virus particles and interfere with the process of infection (8). Whatever the mechanism, there is evidence that terminal hydrophobic groups may stabilise certain interactions of oligonucleotides and thereby increase the antiviral effect (7,8,11,12,13).

There is a great deal of interest in oligonucleotides *anti*-HIV activity and the region around the start codon of the *rev* gene of HIV-1 is a potential target for antisense drug therapy (14). The phosphorothioate 28 mer oligonucleotide Srev is complementary to a region from the 2nd to the 29th bases of the rev gene of HIV-1.

Srev:d(TCG TCG CTG TCT CCG CTT CTT CCT GCC A)

When present at a concentration of 10 μM it inhibits gag p24 glycoprotein synthesis by 50% in chronically infected H9 IIIB cells (14). Inhibition of rev protein synthesis leads to extensive splicing of the full-length HIV m-RNA transcript thus causing a switch from the synthesis of structural proteins to regulatory proteins. The degree of inhibition of the normally abundant p24 glycoprotein therefore gives a convenient measure of the efficacy of *anti*-rev antisense oligonucleotides. Cellular uptake of Srev is not good (4) and this is an obvious area in which significant improvements can be made. In general, the quest for oligonucleotides with improved antisense activity should take into account the problem of transport through cell membranes. Here we describe the synthesis of oligonucleotides with terminal lipophilic groups which are designed to improve cellular uptake. Ultraviolet melting studies on duplexes formed between phosphorothioate oligonucleotides containing lipophilic end-groups and complementary DNA strands have been carried out and several compounds have been evaluated for their ability to inhibit the proliferation of HIV-1 in chronically infected lymphoblastoid T-cells. Results of anti-HIV testing will be reported separately.

RESULTS AND DISCUSSION

Synthesis of hydrophobic monomers and incorporation into oligonucleotides

The solid-phase synthesis of a series of modified phosphorothioate oligonucleotide analogues of Srev bearing the following terminal hydrophobic moieties has been achieved: (i) 3′-and 5′-cholesteryl with and without spacer arms; (ii) 1,2-di-O-hexadecyl-glyceryl; (iii) 5′- and 3′- hexadecyl; (iv) 5′-octadecyl bearing a hydroxyl group on C2; and (v) the 5′-(1-adamantylethyl) group. Oligonucleotides bearing such groups have been studied

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previously in various biological systems and according to published work (5,11,12,13) they should be taken up by cells more readily than standard phosphorothioates. This is particularly true of those containing groups (i) and (ii) which are constituents of cell membranes (17) and oligonucleotides bearing these groups may be actively transported into cells by low density lipoproteins. All lipophilic groups in this study were attached to the oligonucleotides during solid-phase synthesis by the phosphoramidite method. This strategy is more practical and convenient for large scale oligonucleotide synthesis than carbodiimide coupling reactions (5) or oxidative amination (7). It also gives higher yields than the H-phosphonate method (11).

Cholesterol monomers

Previously cholesterol has been attached directly to the 5′-phosphate of oligonucleotides either post-synthetically (5) or during oligonucleotide synthesis using H-phosphonate chemistry (8). It has also been joined to the 3′-phosphate *via* a short carboxyaminoethyl linker (7) or a rigid 5-(hydroxymethyl)-(3R-*trans*)-3-pyrrolidinol linker (9) or as a carbamate at the N(6)-position of adenosine linked to CPG (6). The nature and position of attachment of cholesterol may influence the ability of the modified oligonucleotide to transverse cell membranes or to hybridise efficiently to mRNA. Therefore, in this study we elected to attach cholesterol to the 3′- and 5′-ends of oligonucleotides by linkers of various lengths.

In the first example, reaction of cholesterol [**1a**] with 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite yielded the monomer [**1b**] which was used for direct attachment of cholesterol to the 5′-end of oligonucleotides during solid-phase synthesis. Solid-phase sulphurisation of the phosphite group between the 5′-cholesterol and the adjacent thymidine with tetraethyl thiuram disulphide (TETD) was unsuccessful, presumably due to extreme steric hindrance. However, treatment with iodine in tetrahydrofuran and pyridine resulted in clean oxidation to give a phosphotriester linkage. The solid-phase synthesis of 5′-Ch-Srev proceded smoothly and the product contained the correct ratio of phosphorothioate to phosphate groups (27:1) as confirmed by 600MHz ^{31}P n.m.r. A monomer was also prepared for the attachment of cholesterol to the 5′-end of oligonucleotides *via* a hexamethylene spacer. Thus, reaction of cholesterol-3-chloroformate [**1c**] with aminohexanol gave the cholesterol derivative [**1d**] with a carbamate linkage, a hexyl chain and a free terminal hydroxyl group. Phosphitylation of [**1d**] yielded the phosphoramidite monomer [**1e**] which was dissolved in dichloromethane and used to synthesise 5′-Ch-C6-Srev. Sulphurisation of the unhindered phosphite group at the 3′-side of this monomer proceeded smoothly. For attachment of cholesterol to the 3′-end of oligonucleotides, tosylation of cholesterol and reaction of the tosylate [**1f**] with the sodium salt of solketal gave one major product, the solketal derivative of cholesterol [**1g**] in reasonable yield. The n.m.r. spectrum of [**1g**] is consistent with a diastereomeric mixture due to the use of racemic solketal. The spectrum also reveals that elimination of the tosyl group to give [**1g**] proceeds principally with retention of configuration, as the proton in the 3-position of cholesterol is axial, with large coupling constants (J=11.3Hz) to neighbouring axial cholesterol protons. We were unable to resolve the diastereomers on t.l.c.. Deprotection of the acetonide gave [**1h**] which was dimethoxytritylated to give [**1j**] and succinylated to give [**1k**]. The succinate [**1k**] was used to prepare cholesterol-CPG which was employed in the synthesis of 3′-Ch-Srev. Sulphurisation of the phosphite group on the 5′-side of this monomer during oligonucleotide synthesis was not possible, but prolonged oxidation with iodine (1 hour) was successful and did not give undesirable side-reactions. The glyceryl backbone has been used previously for the attachment of multiple reporter groups to oligonucleotides (22).

Other lipophilic monomers

The 1,2-Di-O-hexadecyl-3-glyceryl group, when attached to the 5′-end of oligonucleotides with normal backbones has been shown to greatly increase oligonucleotide uptake and antiviral activity in L929 cells infected with VSV (11). Previously the group was added as an H-phosphonate monomer but in the present study we decided to use phosphoramidite chemistry throughout in order to obtain higher yields of oligonucleotides. Thus, 1,2-di-hexadecyl glycerol was converted to the monomer [**2**] for use in oligonucleotide synthesis. Addition of the monomer [**2**] to the 5′-terminus of the oligonucleotide chain during solid-phase synthesis was followed by an iodine oxidation. Thus, 5′-$(C16)_2$-Glyc-Srev was readily prepared by this method. Monomers bearing a C16 hydrocarbon chain were prepared and attached to oligonucleotides as follows: The monomer [**3b**] was prepared by dimethoxytritylation and phosphitylation of 1, 2-octadecanediol and was added to the 5′-end of Srev to give C18-Srev. This monomer and the phosphoramidite [**4b**], can, if desired, be added at any position in an oligonucleotide chain, or several units can be added to the 5′-end of the oligonucleotide,

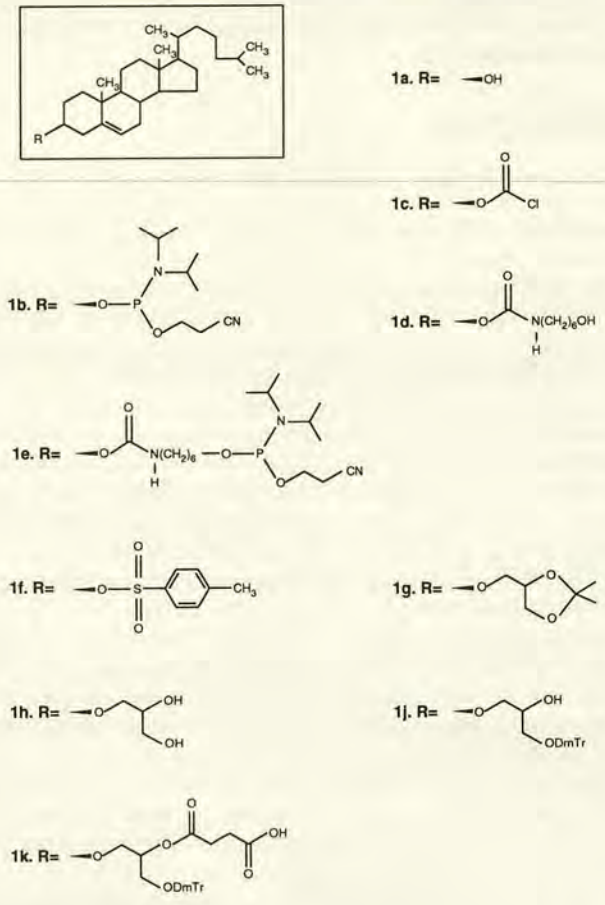


Figure 1. Cholesterol attachment to oligonucleotides.

although this has not yet been done. When [**4b**] is incorporated within an oligonucleotide, the entire polyalkyl chain forms part of the backbone, whereas in the case of [**3b**] an ethylene chain is incorporated in the backbone and a C16 hydrocarbon chain extends from the side. A 3′-C16 hydrocarbon chain can be added to oligonucleotides using CPG functionalised with the succinate [**4c**]. Thus 5′-C16-Srev, 3′-C16-Srev and 5′,3′-C16-Srev were prepared using [**4b**] and [**4c**]. An ethyl-spaced adamantane phosphoramidite [**5**] was prepared by phosphitylation of (1-adamantane)ethan-2-ol. This was used to add an adamantylethyl group to the 5′-end of Srev to give 5′-AdaC2-Srev. It was apparent from reversed-phase H.P.L.C. that the adamantylethyl modified oligonucleotide was much less lipophilic than the other modified oligonucleotides prepared in this study.

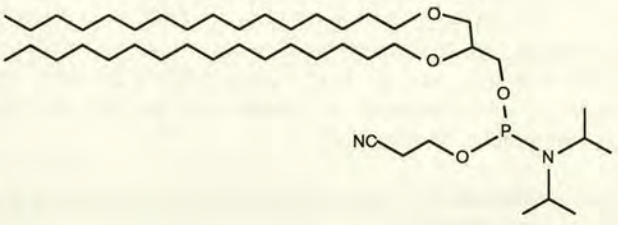
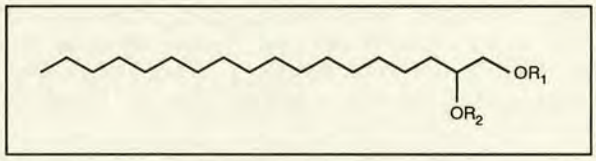


Figure 2.



3a. R_1= DmTr, R_2=H

3b. R_1= DmTr, R_2=

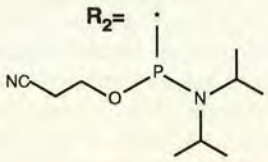
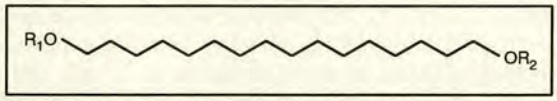
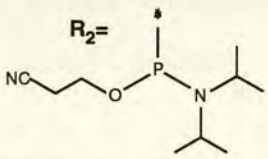


Figure 3.



4a. R_1= DmTr, R_2=H

4b. R_1= DmTr, R_2=



4c. R_1=DmTr, R_2=

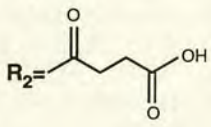


Figure 4.

Synthesis of oligonucleotides with cleavable lipophilic groups

Oligonucleotides attached to lipophilic groups may be sequestered in cell membranes and could therefore become unavailable for binding to viral m-RNA. To address this potential problem we synthesised a number of oligonucleotides attached to lipophilic groups by a tetrathymidine (T_4) linker. Normal phosphodiester bonds are degraded by nuclease enzymes in cells and serum, so the T_4 linker will be slowly cleaved to release the oligonucleotide from the lipophilic moiety *in vivo*. It is possible that a biodegradable linker of this type might confer certain advantages to antisense oligonucleotides.

Ultraviolet oligonucleotide denaturation studies

Terminal lipophilic groups have variously been reported to increase (9,16), decrease (7,11,15,) or to have no effect (7) on oligonucleotide duplex stability. We have measured the ultraviolet melting temperatures of several lipophilic Srev derivatives hybridised to a complementary DNA template with four overhanging nucleotides at the 3′- and 5′-ends of the template corresponding to part of the *rev* gene. The overhangs in the template strand are included to give a closer approximation to the situation *in vivo* where the antisense oligonucleotide hybridises to a much longer strand of m-RNA. This is an important consideration, as the effects of the lipophilic groups cannot be properly evaluated unless the overhangs are present. The ultraviolet melting temperature of the underivatised Srev 28mer hybridized to the complementary DNA 36mer template was determined over a 20-fold concentration range and used as a control. In all cases we found that the stability of the duplex was essentially unaffected by the presence of lipophilic groups (Table 1). The discovery that such bulky groups do not destabilise nucleic acid duplexes is important in the context of hybridisation to mRNA.

CONCLUSION

A number of phosphorothioate oligonucleotide derivatives of Srev have been synthesised with lipophilic groups covalently attached to their 5′- and/or 3′-termini. Several such compounds have been tested for their ability to inhibit the proliferation of HIV-1 virus in chronically infected H9 cells and the results of these studies will be published shortly.

EXPERIMENTAL

All solvents were of analytical grade. Alcohol free dichloromethane was prepared by distillation from CaH_2, anhydrous tetrahydrofuran was purified by distillation from sodium/benzophenone, N,N-dimethylformamide by fractional distillation from 4A molecular sieve and pyridine by distillation from CaH_2. Triethylamine and N,N-diisopropylethylamine were dried over CaH_2. Anhydrous acetonitrile was purchased from Applied Biosystems, 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite from Fluka, 1,2-octadecanediol from Lancaster

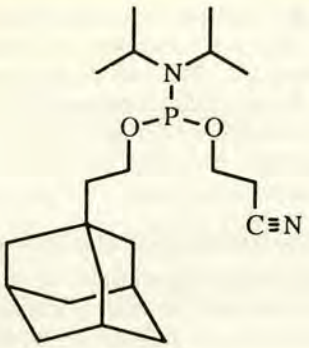


Figure 5.

Table 1. Ultraviolet melting temperatures of hydrophobic derivatives of Srev hybridised to a DNA template

Sequence	Melting Temperature at 5.5 μM
Srev	341.9°K (68.9°C)
5′-Ch-T4Srev	342.2°K (69.2°C)
5′-ChSrev	342.0°K (69.0°C)
5′-Ch-C6Srev	341.5°K (68.5°C)
5′-C18Srev	341.5°K (68.5°C)
5′-C18-T_4Srev	340.8°K (67.8°C)
5′-$(C16)_2$-GlycSrev	342.7°K (69.7°C)
5′-Ad-C2-Srev	342.2°K (69.2°C)

Conditions: Details in experimental section.
Srev = d(TCG TCG CTG TCT CCG CTT CTT CCT GCC A)-phosphorothioate
DNA Template = d(CCT ATG GCA GGA AGA AGC GGA GAC AGC GAC GAA GAC)

Synthesis and all other chemicals were purchased from Aldrich. ^{1}H-NMR spectra were recorded with Brucker WP-80 (80 MHz) and Brucker WP-200 (200MHz) spectrometers. ^{31}P-NMR spectra (81MHz) were recorded with the latter spectrometer . F.a.b mass spectra (positive ion, thioglycerol matrix) were recorded on a Kratos MS50 TC spectrometer. Oligonucleotide synthesis was performed with an Applied Biosystems 380B DNA synthesiser. Standard cyanoethyl phosphoramidite monomers for DNA synthesis were supplied by Cruachem Ltd and were used at a concentration of 0.1M in anhydrous acetonitrile. Sephadex G25 NAP columns (nucleic acid purification) were supplied by Pharmacia. Flash column chromatography was carried out using silica gel 60 (Fluka) and t.l.c. on silica-gel 60 F_{254}, (Merck) using (A). ethyl acetate:methanol:conc aqueous ammonia. (5:1:1) (B). toluene:diethyl ether(4:1). (C). hexane:diethyl ether (1:1), 1% of triethylamine. (D). toluene:diethyl ether (9:1). (E). toluene:diethyl ether (1:1). (F). hexane:ethyl acetate (1:1). (G). hexane:ethyl acetate (4:1).

Products were visualised on t.l.c. using either ultraviolet absorption at 264nm or phosphomolybdic acid (10%) in ethanol (dark blue colour for oxidisable compounds).

General procedure 1—DMTr protected alcohols

Anhydrous pyridine (5 ml) was evaporated from the primary alcohol (3.0 mmol) which was then redissolved in anhydrous pyridine (5 ml). To this solution was added with stirring 4,4′-dimethoxytrityl chloride (3.6 mmol). After 1 hour the reaction was quenched with methanol (10 ml) and the solvent was evaporated *in vacuo*. The residue was dissolved in dichloromethane (100 ml), washed with saturated KCl (20 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The product was purified by silica-gel flash chromatography.

General procedure 2—Phosphoramidite monomers for DNA synthesis

Anhydrous dichloromethane was twice evaporated from the alcohol (0.5 mmol) which was then redissolved in anhydrous dichloromethane (3 ml). Di-isopropylethylamine (1.5 mmol) and 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite (0.7 mmol) were added dropwise under an atmosphere of argon and after stirring for 1 hour the reaction was quenched with ethyl acetate (40 ml), washed briefly with brine (100 ml), dried (Na_2SO_4) , concentrated *in vacuo* and purified by flash chromatography on silica gel.

General procedure 3—Derivatisation of controled pore glass (CPG) solid support

1. Preparation of the succinate monoester. Succinic anhydride (1.1 mmol) was added in portions over a period of 30 minutes to a stirred solution of the alcohol (1.0 mmol) in anhydrous pyridine (2.5 ml) containing 4-dimethylaminopyridine (0.6 mmol). The reaction was stirred overnight, dichloromethane (15 ml) was added and the mixture was washed with water (15 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The product was purified by flash chromatography on silica gel (system A).

2. Coupling the succinate on to CPG. To a solution of the succinate monoester (0.39 mmol) in anhydrous dichoromethane (2 ml) was added a solution of 4-nitrophenol (0.39 mmol, 54 mg) in anhydrous pyridine (0.1 ml) followed by dicyclohexylcarbodiimide (0.94 mmol, 193 mg). After 10 minutes dicyclohexylurea precipitated from the reaction mixture and after 2.5 hours this was removed by filtration. To the filtrate was added triethylamine (0.1 ml) and a suspension of CPG (0.5 g) in dimethylformamide (2 ml). The mixture was shaken overnight and a bright yellow colour developed due to the release of 4-nitrophenol. The CPG was washed with N,N-dimethylformamide (5×10 ml), dichloromethane (5×10 ml) and diethyl ether (5×10 ml) and then dried *in vacuo.*

3. Capping of CPG. To a suspension of the functionalised resin in anhydrous tetrahydrofuran (4 ml) was added a 2M solution of 1-methylimidazole in tetrahydrofuran (0.75 ml) and a 1M solution of acetic anhydride in tetrahydrofuran (1.5 ml). The mixture was shaken for 2.5 hours and the CPG was collected by filtration, washed successively with tetrahydrofuran (5×10 ml), dichloromethane (5×10 ml) and diethyl ether (5×10 ml) then dried *in vacuo*.

3-Cholesteryl-2-cyanoethyl N,N-diisopropylphosphoramidite [1b]

This compound was prepared from cholesterol [**1a**] and 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite according to general procedure 2. Purification by flash chromatography on silica gel (solvent B) gave [**1b**] (1.09 g, 72.0%), R_f 0.81, (solvent B) F.a.b. mass spectrum: *m/z* 587 (M^++H). ^{1}H-N.m.r data ($CDCl_3$): d 1.14−1.15 (d, J= 2.9Hz, 6H), 1.18−1.19 (d, J=2.9Hz, 6H), 2.60−2.66 (t, J=5.8Hz, 2H), 3.53−3.76 (m, 2H), 3.78−3.86 (m, 2H), all additional signals correspond to the cholesteryl moiety.

Cholesterol-3-(carboxyaminohexan-6-ol) [1d]

Cholesteryl chloroformate [**1c**] (1.9 g, 4.2 mmol) and anhydrous triethylamine (0.43 g, 0.59 ml, 4.25 mmol) were dissolved in anhydrous dichloromethane (3 ml) and added to aminohexanol (0.50 g, 4.27 mmol) which had been coevaporated three times with anhydrous pyridine (7 ml). After stirring for 24 hours, dichloromethane (50 ml) was added and the resulting solution was washed with saturated $NaHCO_3$ (20 ml), saturated KCl (2×20 ml) and water (20 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo* . The residue was dissolved in hexane:ethyl acetate (9:1) and purified by flash silica gel chromatography eluting with a gradient of 0% to 50% ethyl acetate in hexane, to give [1d] as a white solid. (1.07 g, 47%). R_f 0.27 (solvent F). F.a.b mass spectrum: *m/z* 530.45730 [calc. for $C_{34}H_{60}NO_3$: (M^++H), *m/z* 530.45729]. ^{13}C-N.m.r. data: ($CDCl_3$): δ 11.68 (CH_3); 18.54 (CH_3); 19.17 (CH_3); 20.85 (CH_2); 22.40 (CH_3); 22.67 (CH_3); 23.66 (CH_2); 24.11 (CH_2); 25.13 (CH_2); 26.21 (CH_2); 27.84 (CH); 28.05 ($2CH_2$); 29.82 (CH_2); 31.69 (CH, CH_2); 32.38 (CH_2); 35.63 (CH); 35.99 (CH_2); 36.36 (C); 36.80 (CH_2); 38.40 (CH_2); 39.33 (CH_2); 39.54 (CH_2); 40.50 (CH_2); 42.11 (C); 49.79 (CH); 55.92 (CH); 56.48 (CH); 62.46 (CH); 74.02 (CH); 122.30 (CH_3); 139.64 (C); 156.10 (C)

Cholesteryl-3-carboxyaminohexyl-2-cyanoethyl N,N-diisopropylphosphoramidite [1e]

Anhydrous dichloromethane (5 ml) was evaporated twice from cholesterol-3-carboxyaminohexan-6-ol (0.5 g; 9.44 mmol) which was then dissolved in anhydrous dichloromethane (5 ml). Diisopropylammonium tetrazolide (0.08 g; 4.72 mmol) was added slowly with stirring and when this had dissolved 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphordiamidite (0.31 g; 0.33 ml; 1.03 mmol) was added. After 24 hours dichloromethane (50 ml) was added and the solution washed with saturated $NaHCO_3$ (20 ml), saturated KCl (2×20 ml) and water (20 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo*. The residual oil was dissolved in hexane:ethyl acetate (9:1) and purified on a silica gel flash chromatography column which had been pre-equilibrated with hexane:ethyl acetate:triethylamine (100:10:1). Elution with a gradient of 0% to 25% ethyl acetate in hexane gave [**1e**] as a clear oil (0.27 g, 39%). R_f 0.62 (solvent F). F.a.b. mass spectrum: *m/z* 729.55730 [calc. for $C_{43}H_{76}N_3O_4P$: (M^+) *m/z* 729.55731]. ^{31}P N.m.r data ($CDCl_3$) d 147.67 (s).

Cholesterol-3-(4-toluenesulphonate) [1f]

4-Toluenesulfonyl chloride (0.30 mol, 57 g) and cholesterol (0.21 mol, 80 g) were dissolved in anhydrous chloroform (150 ml) and cooled in an ice bath. Anhydrous pyridine (1.0 mol, 80.6 g, 82.1 ml) was added with stirring to maintain the temperature between 0° and 3°C and the reaction was stirred for 16 hours at room temperature. A mixture of ice (200 g) and concentrated HCl (70 ml) was then added and the organic phase was washed with water (100 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The product was twice purified by silica gel flash chromatography eluting with toluene:hexane (8:3) to give [**1f**] (61.4 g, 54%). R_f 0.87 (solvent B). F.a.b. mass spectrum: *m/z* 539 (M^+).

3-(2,3-Isopropylidene-1-glyceryl) cholesterol [1g]

A mixture of *rac*-solketal (0.24 mol, 30 ml), cholesterol-3-(4-toluenesulphonate) (0.013 mol, 7.3 g), and sodium (0.065 mol, 1.5 g) in toluene (10 ml) was heated at 120°C for 4 hours and stirred for a further 16 hours at room temperature. The solution was then washed with water (50 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel (solvent D) to give [**1g**] (3.65 g, 56.2%). R_f 0.41 (solvent D). F.a.b. mass spectrum: *m/z* 499 (M^+). ^{1}H-N.m.r. data ($CDCl_3$): d 1.35 (s, 3H); 1.41 (s, 3H); 3.13−3.21 (tt, *J=11.3Hz*, ax-ax, ax-ax, *J=4.4Hz*, ax-eq, ax-eq, 1H, **3-chol**); 3.40−3.45, (dd + dd, $J_{1',1''}$=9.7Hz, $J_{1',2'}$=6.0Hz, 1H, **H1′**); 3.55−3.60 (dd+dd, $J_{1'',1'}$=9.7Hz, $J_{1'',2'}$=5.6Hz, 1H, **H2′**); 3.70−3.74 (dd+dd, $J_{3',3''}$=8.2Hz, $J_{3',2'}$=6.3Hz, 1H, **H3′**), 4.03−4.07 (dd, $J_{3'',3'}$=8.2Hz, $J_{3'',2'}$=6.3Hz, 1H, **H3′′**), 4.19−4.26 (quin, $J_{2',1',1'',3',3''}$=6.0Hz, 1H, **H2′**), all additional signals correspond to cholesteryl moiety. Compound is a mixture of diastereomers due to the use of racemic solketal.

3-(1-glyceryl) cholesterol [1h]

3-(2,3-Isopropylidene-1-glyceryl) cholesterol (2.92 mmol, 1.46 g) was dissolved in 2N HCl:tetrahydrofuran (1:1) (30 ml) and set aside at room temperature for 2 hours. Absolute ethanol (12 ml) was then added and the solvent was removed *in vacuo*. The residue was redissolved in absolute ethanol (12 ml) and concentrated *in vacuo* to give [**1h**] (1.11 g, 83%), R_f 0.13 (solvent E). ^{1}H-N.m.r data: δ 3.45−3.70 (m, 6H), 3.76−3.83 (m, 1H), all additional signals correspond to the cholesteryl moiety.

3-[1-(4,4′-dimethoxytrityl)-3-glyceryl] cholesterol [1j]

This compound was prepared by reaction of dimethoxytrityl chloride with [**1h**] according to general procedure 1 and was purified by flash chromatography on silica gel eluting with hexane:diethyl ether (2:3) with 1% triethylamine added. (1.49 g, 81.3%). R_f 0.41 (solvent C). mass spectrum: *m/z* 762 (M^++H).^{1}H-N.m.r data ($CDCl_3$) d 3.42−3.77 (m, 5H, glyceryl H-1 and H-3, cholesteryl H-3), 3.78 (s, 6H, OMe), 3.79−3.88 (m, 1H, glyceryl H-2), 6.77−6.86 (m, 4H, trityl), 7.15−7.34 (m, 7H, trityl), 7.39−7.45 (m, 2H, trityl), all additional signals correspond to the cholesteryl moiety.

Derivatisation of CPG with 3-[1-(4,4′-dimethoxytrityl)-2-succinyl-3-glyceryl]cholesterol [1k]

This coupling was achieved by reaction of [**1j**] with succinic anhydride according to general procedure 3 to yield 3-[1-(4,4′-dimethoxytrityl)-2-succinyl-3-glyceryl] cholesterol [**1k**]: 0.43 g (50.3%), R_f 0.46 (solvent A). ^{1}H-N.m.r. ($CDCl_3$): δ 1.75−1.80 (m, 2H), 2.67−2.70 (m, 2H), 3.20−3.24 (m, 2H), 3.76 (s,3H), 3.78 (s, 3H), 6.78−6.85 (m, 4H), 7.14−7.33 (m, 7H), 7.40−7.45 (m, 2H), all additional signals correspond to the cholesteryl moiety.

After coupling the amount of nucleoside covalently linked to CPG was found by dimethoxytrityl analysis to be 23.3 μmol/g

2-cyanoethyl (1,2-di-O-hexadecyl-rac-3-glyceryl)-N,N-diisopropylphosphoramidite [2]

This compound was prepared according to general procedure 2 and was purified by flash chromatography on silica gel (solvent B) to give [**2**] (0.20 g, 58.9%). R_f 0.82 (solvent B). F.a.b. mass spectrum: *m/z* 741 (M^++H). ^{1}H-N.m.r data ($CDCl_3$) d 0.83−0.90 (t, *J*=6.2 Hz, 6H), 1.15−1.16 (d, *J*=1.3 Hz, 6H), 1.18−1.19 (d, *J*=1.3 Hz, 6H), 1.24(s, 30 H), 2.59−2.63 (t, *J*=5.9 Hz, 2H), 3.39−3.64 (m, 7H), 3.80−3.85 (m, 2H).

1-(4,4′-dimethoxytrityloxy) octadecan-2-ol [3a]

This compound was prepared from 1,2-octadecanediol and 4,4′-dimethoxytrityl chloride according to general procedure 1 and was purified by flash chromatography on silica gel eluting with system C. (5.58 g , 90.4%). R_f 0.51 (solvent C). F.a.b. mass spectrum *m/z* 588(M^+).^{1}H-N.m.r. data ($CDCl_3$): δ 0.92−0.98 (t, *J*=5.8 Hz, 3H), 1.34 (s, 30 H), 2.58 (s, 1H), 3.06−3.26 (m, 3H), 3.79 (s, 6H), 6.85−6.90 (d, *J*=8.9 Hz, 4H), 7.22−7.42 (m, 7H), 7.49−7.50 (m, 2H).

2-Cyanoethyl [1-(4,4′-dimethoxytrityloxy) octadec-2-yl] N,N-di-isopropylphosphoramidite [3b]

This compound was prepared from 1-(4,4′-dimethoxytrityloxy)octadecan-2-ol and 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite according to general procedure 2 and was purified by flash chromatography on silica gel eluting with anhydrous hexane:toluene (1:1) with 1% triethylamine. Further purification was performed on a flash column eluting with anhydrous hexane:diethyl ether (1:1).

Yield: 1.14 g (85.0%), T.l.c. (system C), R_f 0.58, 200MHz-^{1}H-NMR, d: 0.84−0.91 (t, J=6.1Hz, 3H), 1.03−1.20(m, 12H), 1.25(s, 30H), 2.35−2.41(t, J=6.7Hz, 2H), 2.57−2.63(t, J=6.1Hz, 2H), 3.10−3.16(m, 3H), 3.54−3.77(m, 2H), 3.78(s, 6H), 3.79−3.87(m, 2H), 6.77−6.85(dd, J=8.9Hz, J=3.9Hz, 4H), 7.18−7.36(m, 7H), 7.43−7.48(m, 2H). Mass Spectrum (+FAB), m/z 789(M^+).

1-(4,4′-dimethoxytrityloxy) hexadecan-16-ol [4a]

This compound was prepared according to general procedure 1 but with 5 equivalents of 1,16-hexadecanediol and 1 equivalent of dimethoxytrityl chloride.

The product was purified by flash chromatography on silica gel eluting with hexane:diethyl ether (1:4) with 1% triethylamine to give [**4a**] (0.58g, 70.1%). R_f 0.18 (solvent C). F.a.b. mass spectrum: *m/z* 560 (M^+). ^{1}H-N.m.r data ($CDCl_3$): d 1.24 (s, 28 H), 2.98−3.02 (m, 2H), 3.62−3.64 (m, 2H), 3.76 (s, 3H), 3.78, (s, 3H), 6.77−6.83 (m, 4H), 7.22−7.34 (m, 7H),7.41−7.47, (m, 2H).

2-Cyanoethyl [1-(4,4′-dimethoxytrityloxy) hexadec-16-yl] N,N-di-isopropylphosphoramidite [4b]

This compound was prepared according to general procedure 2 and was purified by flash chromatography on silica gel eluting with anhydrous hexane:diethyl ether (1:1) with 1% triethylamine. A second purification was performed eluting with hexane:diethyl ether (1:1) to give [**4b**] (0.41g, 68.2%). R_f 0.52 (system C). F.a.b. mass spectrum *m/z* 761 (M^++H). ^{1}H-NMR data ($CDCl_3$): δ 1.24 (s, 28 H ,CH_2), 1.56 (s, 12H, CH_3), 2.61−2.67 (m, 2H, cyanoethyl H-1), 2.98−3.05 (m, 2H, i-Pr CH), 3.51−3.68 (m, 4H, H-1, H-16), 3.78 (s, 6H, OMe), 3.81−3.88 (m, 2H, cyanoethyl H-2), 6.79−6.94 (m, 4H, trityl), 7.18−7.34 (m, 7H, trityl), 7.41−7.45 (m, 2H, trityl).

Derivatisation of CPG with 1-(4,4′-dimethoxytrityloxy) hexadecyl-16-succinate [4c]

This coupling was achieved according to the general procedure 3 to give [**4c**] (0.13 g, 55.5%). R_f 0.43 (solvent A). F.a.b. mass spectrum: *m/z* 659 (M^+). ^{1}H-N.m.r data ($CDCl_3$): d 1.24 (s, 28H), 1.59−1.61 (m, 2H), 2.62−2.65 (m, 2H), 2.98−3.05 (t, *J*=6.5 Hz, 2H), 3.78 (s, 6H), 4.04−4.11 (t, *J*=6.7 Hz, 2H), 6.79−6.85 (m, 4H), 7.18−7.35 (m, 7H), 7.41−7.46 (m, 2H).

After coupling, the amount of nucleoside covalently linked to CPG was found by dimethoxytrityl analysis to be 24.6 μmol/g

1-Adamantylethyl-2-cyanoethyl N,N-diisopropylphosphoramidite [5]

To a solution of 1-adamantylethan-2-ol (0.5 g, 2.77 mmol) in anhydrous tetrahydrofuran (10ml) was added N,N-diisopropylethylamine (4 equiv., 0.11 mol, 1.43 g, 1.93 ml) and 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite (1.1 equiv., 3.05 mmol, 0.72 g, 0.68 ml). After 5 minutes the reaction was quenched with anhydrous ethanol (1 ml) and dichloromethane (15 ml) was added. The mixture was washed with water (20 ml) and saturated KCl (2×20 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo*. The product was purified by flash silica gel chromatography, on a column which had been pre-equilibrated with hexane:ethyl acetate:triethylamine (100:10:1) Elution with hexane :ethyl acetate (9:1) gave [5] as a clear liquid (0.72 g, 69%). R_f 0.70 (solvent G). F.a.b. mass spectrum: *m/z* 379.25142 calc. for $C_{21}H_{36}N_2O_2P$ (M^++H) *m/z* 379.25143]. ^{31}P N.m.r data ($CDCl_3$): d 147.21 (s).

Oligonucleotide synthesis

Three 1.0 mmole scale syntheses were performed for each oligonucleotide using cyanoethyl phosphoramidite chemistry on an ABI 380B DNA synthesiser. For phosphorothioate oligonucleotide synthesis iodine was replaced by tetraethyl thiuram disulphide (TETD) (19). All lipophilic phosphoramidite monomers were used as 0.1M solutions in anhydrous dichloromethane. In order to increase the coupling yields of phosphoramidite monomers [**1b**], [**2**] and [**3b**] above 75%, coupling times were increased from the standard 40 seconds to 4 minutes. In all cases an additional dichloromethane column wash before and after the coupling step was included in the synthesis cycle to prevent precipitation of the monomer and blockage of reagent lines. Sulphurisation was unsuccessful for compounds [**1b**], [**2**] and [**3b**], and for 3′-cholesterol oligonucleotides, so the sulphurisation step was replaced with an iodine oxidation step lasting 10 min. The trityl-ON configuration was used throughout and all oligonucleotides were obtained in quantities in excess of 10 mg after full purification.

Oligonucleotide purification

After deprotection with concentrated ammonium hydroxide, oligonucleotides were dried under a stream of argon, dissolved in H.P.L.C. buffer A (below) and purified by reversed-phase H.P.L.C. using a Brownlee Aquapore Octyl reverse phase column (10 mm×250 mm) with a flow rate of 3 ml/minute and the following gradient:

Time (minutes)	%Buffer B
0	0
3	0
4	15
19*	100*
28	100
29	0
35	0

Buffer A: 0.1M NH_4OAc. *Buffer B:* 0.1M NH_4OAc with 60% acetonitrile.
*For oligonucleotides containing the adamantaneethyl group the step at 19 minutes was omitted to produce a shallower gradient.

The desired products elute at 100% B, several minutes after the failure sequences. Where appropriate the H.P.L.C. purified oligonucleotides were detritylated in 80% acetic acid for 30 minutes. After evaporation to remove the solvent, oligonucleotides were further purified twice by gel-filtration using Sephadex NAP-10 columns (Pharmacia) and were then lyophilised. Purified water (purified by reverse osmosis) was used for all stages of purification after H.P.L.C.

Ultraviolet melting studies

Ultraviolet melting temperatures were determined at 260 nm on a Perkin-Elmer Lambda 15 ultraviolet spectrometer equipped with a Peltier block and controlled by an IBM PS2 microcomputer. A heating rate of 0.9 K per minute was used throughout and the crude data were collected and processed using the PECSS-2 software package. The oligonucleotides were dissolved in a buffer consisting of aqueous sodium chloride (0.1 M), sodium dihydrogen orthophosphate (0.01 M), sodium cacodylate (0.02 M) and EDTA (1 mM) adjusted to pH 7.0 by the addition of sodium hydroxide. Concentrations of normal oligonucleotide templates were determined by dissolving the sample in a buffer consisting of NaCl (0.1 M) and Tris.HCl (10 mM) at pH 8.7 and maintaining this at 37°C in the presence of 0.1 mg of phosphodiesterase 1 and 0.1 mg of alkaline phosphatase until complete digestion gave rise to maximum ultraviolet absorbance (< 1 day). Published ϵ_{260} values of the nucleosides were used as standards (21). As phosphorothioate oligonucleotides are resistant to enzymic digestion, their concentrations were estimated by measuring the U.V. absorbance of the intact oligonucleotide and multiplying this by a factor equivalent to the proportional increase in U.V. absorbance obtained by digestion of a normal oligonucleotide of the same base sequence. Melting temperatures of lipophilic derivatives of Srev hybridised to a normal DNA template with four overhanging bases at both the 3′-, and 5′-end were measured and compared with the ultraviolet melting temperature of underivatised Srev hybridised to the same DNA template. The T_m of Srev was measured at eight points over a 20-fold concentration range (each point measured in triplicate) and thermodynamic parameters were determined by standard methods (20).

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REFERENCES

1. Uhlmann, E., and Peyman, A. (1990),*Chem. Rev.* **90** , 543–584.
2. Agrawal, S., Goodchild, J., Civeira, M.P., Thornton, A.H., Sarin, P., S. and Zamecnik, P.C. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7079–7083.
3. Agris, C.H., Blake, K.R., Miller, P.S., Reddy, M.P., Ts'o, P.O.P. (1986)*Biochemistry* **25**, 6268–6275.
4. Jaroszewski, J.W. and Cohen, J.S. (1991), *Adv. Drug Delivery Revs*, **6**, 235–250.
5. Boutorin, A. S., Gus'kova, L. V., Ivanova, E. M., Kobetz, N. D., Zarytova, V. F., Ryte, A. S., Yurchenko, L. V. and Vlassov, V. V. (1989), *FEBS LETTERS*, **254**, 129–132.
6. Gmeiner, W. H., Luo, W., Pon, R.T. and Lown, J. W. (1991), *Bioorg and Med.Chem. Lett.* **1**, 487–490.
7. Letsinger, R, L., Zhang, G., Sun, D, K., Ikeuchi, T. and Sarin, P. S. (1989), *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6553–6556.
8. Stein, C. A., Pal, R., De Vico, A.L., Hoke, G., Mumbauer, S., Kinstler, O., Sarngadharan, M. G. and Letsinger, R. L. (1991), *Biochemistry*, **30**, 2439–2444.
9. Reed, M.W., Adams, A.D., Nelson, J.S. and Meyer, R.B. Jnr. (1991), *Bioconjugate Chem.*, **2**, 217–225.
10. Will, D.W. and Brown, T. (1992), *Tetrahedron Lett.* **33**, 2729–2732.
11. Shea, R. G., Marsters, J. C. and Bischofberger, N. (1990), *Nucleic Acids Res.* **18**, 3777–3783.
12. Saison-Behmoaras, T., Tocque, B., Rey, I., Chassignol, M., Thuong, N.T. and Helene, C. (1991), *EMBO J.*, **10**, 1111–1119.
13. Kabanov, A. V., Vinogradov, S. V., Ovcharenko, A. V., Krivonos, A. V., Melik-Nubarov, N. S., Kiselev, V. I. and Severin, E. S. (1990), *FEBS LETTERS*, **259**, 327–330.
14. Matsukura, M.; Zon, G.; Shinozuka, K.; Robert-Guroff, M.; Shimada, T.; Stein, C.A.; Mitsuya, H., Wong-Staal, F., Cohen, J.S., Broder, S. (1989), *Proc. Natl. Acad. Sci. USA* , **86** , 4244–4248.
15. Kempe, T., Sundquist, W.I., Chow, F. and Hu, S-L. (1985), *Nucleic Acids Res.*, **13**, 45–57.
16. Jaeger, A., Levy, M.J., and Hecht, S.M. (1988), *Biochemistry*, **27**, 7237–7246.
17. Crews, F. T., Mc Elhaney, M. R., Klepner, C. A., and Lippa, A. S. (1988), *Drug Dev. Res.* **14**, 31–44.
18. Majumdar, C., Stein, C.A., Cohen, J. *et al.* (1989), *Biochemistry*, 28, 1340–1346.
19. Vu, H., and Hirshbeim, B.L. (1991), *Tetrahedron Lett*, 32, 3005–3008.
20. Aboul-ela, F., Koh, D. & Tinoco, I. Jr. (1985), *Nucleic Acids Res.* **13**, 4811–4824.
21. Jones, R. A. (1984), Chapter 2, pp 23–34 in *'Oligonucleotides, a Practical Approach.'* Ed. Gait, M. J. I.R.L. press Ltd.
22. Misiura, K., Durrant, I., Evans, M.R. and Gait, M.J. (1990), *Nucleic Acids Res*, **18**, 4345–4354.